APPENDIX A

NONIONIC OLIGONUCLEOTIDE ANALOGS AS NEW TOOLS FOR STUDIES ON THE STRUCTURE AND FUNCTION OF NUCLEIC ACIDS INSIDE LIVING CELLS

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ABSTRACT

Two types of nonionic oligonucleotide analogs, deoxyribonucleotide alkyl phosphotriesters and deoxyribooligonucleoside methylphosphonates, have been synthesized to serve as selective inhibitors of cellular nucleic acid function. The backbones of these analogs are resistant to nuclease hydrolysis and the analogs are taken up by mammalian cells and certain bacterial cells in culture. Sequence specific analogs inhibit tRNA aminoacylation and translation of mRNA in both mammalian and bacterial cell-free systems in a specific manner as a result of oligomer binding to complementary sequences of the target nucleic acid. These analogs also inhibit cellular protein synthesis and growth of living cells. Selective inhibition of bacterial versus mammalian cell growth is observed with a methylphosphonate oligomer complementary to the Shine-Dalgarno sequence of 16S rRNA. Methylphosphonate complementary to the 5'-end of U_1 RNA and to the donor splice site of SV40 large T antigen pre-mRNA inhibit T-antigen production in SV40-infected cells.

INTRODUCTION

Studies on nucleic acid analogs possessing modified internucleoside linkages have made important contributions to our understanding of nucleic acid conformation and have provided materials for a variety of bi chemical and biological studies (1-12). We have studied two types of nonionic oligonucleotides, oligonucleotide alkylphosphotriesters and oligodeoxyribonucleoside methylphosphonates, whose structures are shown in Figure 1. The 3'-5' linked internucleotide bonds of these analogs closely resemble the size and geometry of the nucleic acid phosphodiester bond. However, since the sugar-phosphate backbones of these analogs are electroneutral, the analogs have unique physical and biological properties. These properties include (1) their ability t f rm stable hydr gen-b nded complexes with c mplementary polynucleotides; (2) their resistance to hydrolysis by nucleases; and (3) their ability to be taken up intact by mammalian and certain bacterial cells.

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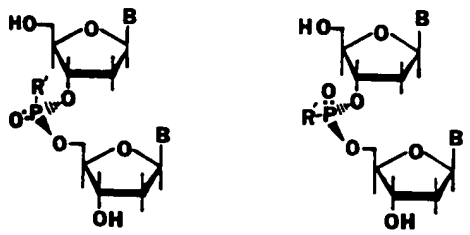
EXHIBIT A

Figure 1



The properties of nonionic oligonucleotides suggest they c uld specifically bind to single-stranded regions of cellular nucleic acid As a consequence of binding, the analogs may inhibit the functi n or expression of cellular or viral nucleic acids in a selective manner. We have tested this possibility by examining the effects of sequence-specific analogs on aminoacylation of tRNA, translation of mRNA and splicing of pre-mRNA both in the test tube and in living cells. The results of our experiments suggest nonionic oligonucleotides may inde be designed to specifically control nucleic acid function. We will first briefly describe the physical properties of these anal gs and then describe experiments designed to examine the biochemical and biological properties of these molecules.

PHYSICAL PROPERTIES

Dideoxyribonucleoside methyl and ethyl phosphotriester [dNp(R)N] and methylphosphonate [dNpN] dimers occur as a pair of diastereoisome: which differ in their configuration about the phosphorous atom (Figur 1). The effects of the phosphotriester group configuration on dimer conformation were studied by NMR (13). The detailed conf rmations of methylphosphonate diastereoisomers were studied by circular dichroism and by NMR (14,15). The absolute configuration of d-ApT has been determined by X-ray crystallography (16) while that of d-ApA has been assigned by NMR nuclear Overhauser enhancement experiments (15).

As shown by CD and ^{1}H NMR, dinucleoside methylphosphonates ad pt stacked conformations in aqueous solution similar to those of dinucle side monophosphates. The conformation of the sugar-phosph nate backbone as defined by the puckering of the deoxyribofuranose rings and th rotation about ψ, ϕ, and ϕ' is very similar to that of the dinucleosid monophosphates. The base stacking of d-ApA-S-isomer is slightly great er than that of the R isomer and is almost identical to that f

d-ApA. These differences in conformation may result from the differences in solvation of the two isomers. Thus the hydrophobic methyl group of the S-isomer of d-ApA is located near the hydrophobic base stacking region which would tend to stabilize base stacking interactions in the dimer. In contrast, the methyl group in the R-configuration is directed away from the base stacking region and may be expected to destabilize stacking interactions.

In some cases, the configuration of the alkylphosphotriester group or the methylphosphonate group can influence interactions of nonionic oligomers with complementary polynucleotides. For example, d-ApA forms 2U:1A triple stranded complexes with polyuridylic acid (Table I).

TABLE I. Interaction of Oligonucleoside Methylphosphonates with Complementary Polynucleotides (a)

Oligomer	Tm with poly(rU) (2U:1A) (°C)	Tm with poly(dT) (2T:1A) (°C)
d-ApA R-isomer	15.4	18.7
S-isomer	19.8	18.4
d-ApApA	33.0	36.8
d-ApApApA	43.0	44.5
d-ApA	7.0	9.2
d-ApApApA	32.0	35.5

(a) 5×10^{-5} M total (nucleotide), 10 mM Tris and 10 mM $MgCl_2$, pH 7.5.

The melting temperature of the S isomer complex is approximately 40 higher than that of the R isomer complex, while both complexes have Tm's higher than that of d-ApA·poly U (14). Similar increases in Tm are seen for complexes between d-ApApA and d-ApApApA and poly U and poly(dT)(17). The sharpness of the melting curves indicates the various diastereoisomers form complexes of similar stability.

More dramatic effects of configuration are seen for oligothymidylate ethylphosphotriesters (18) and oligothymidylate methylphosphonates (Table II). The triester d-[Tp(Et)$_7$]T, which consists of 2^7 diastereoisomers, forms a 1:1 complex with poly (dA), which displays a rather broad melting curve. The octamer triester does not bind to poly(dA)·poly(dT) and interacts with poly(rA) only at low temperature. Similar results were obtained for the methylphosphonate, d-(Tp)$_8$T. These results suggest is mers of different backbone configuration form

TABLE II. Interaction of Nonionic Oligothymidylates with Complementary Polynucleotides

Oligomer	Tm with poly(dA)	Tm with poly(rA)
d-[Tp(Et)]$_7$T (a)	18° (1T:1A)	<0°
d-(Tp)$_8$T (b)	22° (1T:1A)	<0°
d-Tp(TpTp)$_4$T (b)		
Isomer 1	33.5° (1T:1A)	19.5° (1T:1A)
Isomer 2	2° (2T:1A)	0°
d-(Tp)$_9$T (b)	22.5 (1T:1A)	18.0° (1T:1A)

(a) 1 x 10^{-4} M total [nucleotide], 0.15 M NaCl and 0.04 M potassium phosphate, pH 6.9.
(b) 3.5 x 10^{-5} M total [nucleotide], 0.10 M sodium cacodylate, pH 6.8.

complexes of unique stability with poly(dA) and poly(rA). This conclusion was confirmed by examining the alternating methylphosphonate/phosphodiester oligothymidylate analog, d-Tp(TpTp)$_4$T where the configuration of each methylphosphonate linkage is the same throughout the backbone of the oligomer and is denoted as type 1 or type 2 (19). As shown in Table II, the oligomer with type 1 configuration forms stable complexes with both poly(dA) and poly(rA) while that with type 2 configuration forms a 2U:1A complex with poly(dA) and no complex with poly(rA).

The Tm values of nonionic oligonucleotide/polynucleotide complexes are not affected by changes in salt concentration. This effect results from the reduced charge repulsion between the nonionic backbone of the oligomer and negatively charged sugar-phosphate backbone of the polynucleotide. The lack of charge repulsion also explains the increased stabilities of nonionic oligonucleotide/polynucleotide complexes versus those of oligonucleotide phosphodiester/polynucleotide complexes.

SEQUENCE-SPECIFIC INHIBITION OF CELL-FREE AMINOACYLATION AND PROTEIN SYNTHESIS BY NONIONIC OLIGONUCLEOTIDES

Sequence-specific nonionic oligonucleotides form hydrogen-bonded complexes with the -ACCA- amino acid accepting stem and anticodon loop regions of tRNA (17,20,21). For example, the binding constants of tritium-labeled $G^{m}_{p}(Et)G^{m}_{p}(Et)U$ with $tRNA^{Phe}$ yeast, unfractionated

$tRNA_{E.coli}$ and unfractionated $tRNA_{E.coli}$ lacking the 3'-CpA terminus and d-GpGpT with $tRNA_{E.coli}$ are shown Table III.

TABLE III. Interaction of Nonionic Oligonucleotides with Transfer RNA(a)

Oligomer	Temp. (°C)	K (M^{-1}) $tRNA^{Phe}$ yeast	K (M^{-1}) $tRNA_{E.coli}$	$tRNA_{E.coli}$-CA
$G^{m}p(Et)G^{m}p(Et)U$	0	3,100	9,300	1,600
	25	3,100	1,900	-
	37	1,700	2,000	-
$G^{m}pG^{m}pU$	0	63,500	103,000	4,000
	25	5,300	12,300	-
	37	750	1,100	-
d-GpGpT	0	-	1,000	-
	25	-	200	-
	37	-	100	-

(a) The binding constants were measured by equilibrium dialysis in 0.01 M NaCl, 10 mM $MgCl_2$, 10 mM Tris, pH 7.5.

The binding constants of the nonionic oligomers show relatively small changes over the temperature range studied, while that of the diester, $G^{m}pG^{m}pU$, dramatically diminishes with increasing temperature. This effect may be due to self-aggregation of the nonionic oligomers at low temperatures, which would result in a decreased apparent binding to the tRNA. The apparent association constants of d-GpGpT are significantly less than those of 2'-O-methylribooligonucleotide ethyl phosphotriester. This difference may reflect overall differences in the conformation of the deoxyribo- versus 2'-O-methylribo backbones of these oligomers. Removal of the 3'-CpA nucleotides from unfractionated $tRNA_{E.coli}$ by treatment with snake venom phosphodiesterase results in a dramatic reduction of the binding constants for the oligomers. This indicates the major binding site is indeed the 3'-amino acid accepting end of the tRNA. The observed residual binding may be due to binding t other complementary single-stranded regions of the unfractionated tRNA.

As shown in Table IV, sequence-specific nonionic oligonucleotides inhibit cell-free aminoacylation of tRNA (17,21,22). Oligodeoxyadenosine methylphosphonates and the parent diester, d-ApApApA selectively inhibit cell-free aminoacylation of $tRNA^{Lys}_{E.coli}$. The extent of inhibition is temperature dependent and parallels the ability of the oligomers to bind to poly(rU) (Table I). These observations and the previously dem nstrated interaction of r-ApApApA with $tRNA^{Lys}_{E.coli}$ (23) suggest the inhibition is a consequence of olig mer binding t the -UUUU- anticodon lo p of the tRNA. The lower extent of inhibition

observed with d-ApApGpA is consistent with this explanation, since interaction of this oligomer with the anticodon loop would inv lve formation of a less stable G·U base pair.

TABLE IV. Effects of Nonionic Oligonucleotides on Cell-Free Aminoacylation of Unfractionated $tRNA^{Lys}_{E.coli}$ (a)

	% Inhibition				
	Phe	Leu	Lys		
Oligomer	0°C	0°C	0°C	22°C	37°C
d-ApA	6	0	7	-	-
d-ApApA	9	0	62	15	0
d-ApApApA	9	12	88	40	16
d-ApApGpA	12	12	35	0	-
d-GpGpT	31	5	34	9	15
$G^{m}p(Et)G^{m}p(Et)U$	39(b)	-	-	-	-
d-ApApApA	0	7	71(c)	15(c)	-
d-GpGpT (400 μM)					

(a) Reactions were carried out in 100 mM Tris-HCl, pH 7.4, 10 mM $Mg(OAc)_2$), 5 mM KCl, 2 mM ATP, 4 μM ^{3}H-labeled amino acid, 2 μM tRNA using unfractionated E.c li aminoacyl synthetase in the presence of 50 μM oligomer.
(b) 37°C.
(c) [oligomer] = 100 μM.

Since the anticodon loop of $tRNA^{Lys}_{E.coli}$ forms part of the synthetase recognition site (24,25), inhibition of aminoacylation by the methylphosphonates could result from a reduction in the affinity of the synthetase for the $tRNA^{Lys}$-oligonucleotide complexes. Alternatively, oligomer binding to the anticodon loop may induce conformati nal changes in the tRNA, thus leading to a lower rate and extent of amin acylation. The greater inhibiti n by d-ApApApA versus d-ApApApA may be a c nsequence of greater binding f the methylphosph nate analog to the anticodon loop or to a decreased ability f the synthetase t displace the n ni nic oligonucleotide anal g.

Both phenylalanine and lysine aminoacylation are inhibited by the d-GpGpT at 0°, while little effect is observed on leucine aminoacylation. These differences may reflect differences in the ability of the oligomer to bind to the -ACC- ends of the tRNAs. Inhibition of lysine aminoacylation by d-GpGpT is very temperature dependent while G^{m}_{p}(Et)G^{m}_{p}(Et)U effectively inhibits phenylalanine aminoacylation even at 37°C. This behavior parallels the ability of the oligomers to bind to tRNA (Table III).

As shown in Table V, oligodeoxyribonucleoside methylphosphonates effectively inhibit polypeptide synthesis in cell-free systems derived from E.coli and rabbit reticulocytes (17). Poly(U)-directed polyphenylalanine synthesis is inhibited by oligodeoxyadenosine analogs in both cell-free systems. The extent of inhibition reflects the stabilities of the oligomer/poly(U) complexes (Table I). Thus, d-ApApGpA, which forms a less stable complex with poly(U), is 4.5-fold less effective than d-ApApApA. These observations suggest inhibition results from complex formation between the poly(U) message and the oligomers. It is unlikely inhibition results from non-specific interactions of the oligodeoxyadenylate analogs with protein components of the translation systems, since no inhibition of globin mRNA translation by these analogs is observed in the reticulocyte system.

TABLE V. Effects of Oligonucleoside Methylphosphonates on Bacterial and Mammalian Cell-Free Protein Synthesis at 22°C

	% Inhibition		
	E. coli	Rabbit Reticulocyte	
Oligomer	Poly(U) Directed(a)	Poly(U) Directed(a)	Globin mRNA Directed (b)
d-ApA	20	-	-
d-ApApA	84	81	-
d-ApApApA	100	77	0
d-ApApGpA	22	-	0
d-CpCpApT	-	-	61(c)
d-GpCpApCpCpApT	-	-	40(d)
d-$(Tp)_5$T	-	-	0(e)
d-ApApApA	13	18	0

(a) [poly(U)] = 360 μM in U; [oligomer] = 175-200 μM in base.
(b) [oligomer] = 200 μM in base.
(c) [oligomer] = 246 μM in strand.
(d) [oligomer] = 289 μM in strand.
(e) [oligomer] = 300 μM in strand.

Although d-ApApA and the phosphodiester d-ApApApA form c mplexes with poly(U) which have very similar Tm values (Table I), the methylphosphonate analog more effectively inhibits translation. This effect may result from a decreased ability of the ribosome to displace the nonionic methylphosphonate oligomer from the poly(U). Alternatively the phosphodiester oligomer may be susceptible to degradation by nucleases in the cell-free translation systems.

d-CpCpApT is complementary to the -AUGG- initiation codon region of globin mRNA and to the anticodon region of $tRNA^{his}$. d-GpCpApCpCpApT and d-$(Tp)_5$T are complementary respectively to the initiation cod n regions and poly(A) tails of rabbit α and β globin mRNA. B th d-CpCpApT and d-GpCpApCpCpApT effectively inhibit incorporation of of [^{3}H]-leucine into globin, while d-$(Tp)_5$T has no effect on translatio The greater inhibition by d-CpCpApT could be due to oligomer binding tc a number of complementary sequences along the coding region of the globin mRNA as well as to the anticodon region of $tRNA^{his}$. The lack f inhibition by d-$(Tp)_5$ suggests potential binding to the poly(A) tail of globin mRNA does not affect translation and also shows the bserved inhibition is sequence specific.

Specific inhibition of bacterial protein synthesis can be affected by disrupting the interaction between ribosomal RNA and mRNA (26). Oligonucleoside methylphosphonates were synthesized whose base sequences are complementary to the Shine-Dalgarno sequence (-ACCUCCU-) found at the 3'-end of bacterial 16S rRNA. This sequence is required for binding of the 40S ribosomal subunit to bacterial mRNA. A similar sequence is lacking in eukaryotic 18S rRNA, and ribosome binding m st likely begins by recognition of the 5'-cap site of eukaryotic mRNAs.

The interactions of d-ApGpGpApGpGp[^{3}H]-T and d-ApGpGp[^{3}H]T with 70S ribosomes were studied by equilibrium dialysis. The heptamer has a high apparent binding constant which diminishes with increasing tempera ture (4.67×10^5 M^{-1} at 0°C; 1.72×10^5 M^{-1} at 22°C; 2.0×10^4 M^{-1} at 37°C). The tetramer has an approximately ten-fold lower binding constant (1.44×10^4 M^{-1} at 22°C). As shown in Table VI, d-ApGpGpApGpG and d-ApGpGpApGpGpT exhibit significant inhibitory activities when MS-2 RNA is the message, but show less effect on poly(A)-directed polyphenyl alanine or poly(A)-directed polylysine synthesis.

TABLE VI. Effects of Deoxyribonucleoside Methylphosphonates on Cell-Free Translation in an E. Coli System

Oligomer	Conc. μM	% Inhibition Poly(U)(a) 22°C	Poly(U)(a) 37°C	Poly(A)(b) 22°C	Poly(A)(b) 37°C	MS2 RNA 22°C
d-ApGpGp	100	8	0	0	0	5
d-ApGpGpT	100	-	-	-	-	0
d-ApGpGpApGpGp	12.5	-	-	-	-	45
	25	0	0	0	0	75
	50	19	0	29	14	88
	100	39	18	80	27	-
d-ApGpGpApGpGpT	25	0	0	0	0	77

(a) 260 μM in UMP residues.
(b) 225 μM in AMP residues.

Inhibition is temperature and concentration dependent. The shorter oligomers d-ApGpGp and d-ApGpGpT show little or no inhibitor activity, even at high nucleotide concentrations. In contrast to their effects on the E.coli system, neither d-ApGpGpApGpGp or d-ApGpGpApGpGpT show appreciable inhibitory effects on translation of globin mRNA in a cell-free reticulocyte system (at 100 μM and 22°C, 16% and 17%, respectively).

These results strongly suggest specific inhibition of MS2 RNA translation in the E.coli cell-free system is a consequence of oligomer binding to the Shine-Dalgarno sequence of 16S rRNA. This binding prevents the 40S ribosome from binding to the mRNA. Because the synthetic mRNAs, poly(U) and poly(A) lack specific initiation sites, much lower inhibition of translation by the oligomers is observed. Although the 3'-end sequences of 18S rRNA and 16S rRNA are similar, 18S rRNA specifically lacks the -CCUCCU- sequence found in 16S rRNA. Thus, the oligonucleoside methylphosphonates cannot form stable complexes with the 18S rRNA of reticulocyte ribosomes.

UPTAKE OF NONIONIC OLIGONUCLEOTIDES BY LIVING CELLS

The internucleotide bonds of alkylphosphotriesters and methylphosphonate oligomers are completely resistant to hydr lysis by exo- and endonucleases and nuclease and esterase activities f und in mammalian sera (13,17,19,20,21). Olig mer analogs which have been incubated with mammalian cells in culture are recovered c mpletely intact fr m the culture medium. Tritium-labeled oligonucleotide ethylph sph triesters

and oligonucleoside methylphosphonates are readily taken up by mammalian cells in culture. In the case of $G^{m}_{p}(Et)G^{m}_{p}(Et)[^{3}H]$-U and d-$[Tp(Et)]_{n}[^{3}H]T$ (n = 1,4,6), the oligomers are rapidly taken up by transformed Syrian hamster fibroblasts (21; Miller and Jayaraman, unpublished results) and subsequently metabolized. Analysis by chr matography of the radioactivity recovered from cell lysates after a 2 hr incubation with $G^{m}_{p}(Et)G^{m}_{p}(Et)[^{3}H]$-U shows 27% of the label ccurs in th trinucleotide species $G^{m}_{p}(Et)G^{m}_{p}(Et)U$, $G^{m}_{p}G^{m}_{p}(Et)U$, $G^{m}_{p}(Et)G^{m}_{p}U$ and $G^{m}_{p}G^{m}_{p}U$, 28% is incorporated as uridine or cytidine in high-molecular-weight RNA, and the remainder is found in various mono- and dimeric species. These results suggest the triester is taken up intact by the cells, deethylated, and the resulting phosphodiester linkages may then be further hydrolyzed by nucleases.

The uptake of oligonucleoside methylphosphonates by transformed Syrian hamster fibroblasts is quite different from that of the ligonucleotide ethylphosphotriesters (17). The rate and extent of uptake is consistent with passive diffusion of the oligomer across the cell membrane. Thus, after 1.5 hr., the calculated intracellular concentration is ∿177 μM when cells are incubated with 100 μM d-T<u>p</u>[^{3}H]T. Both d-T<u>p</u>[^{3}H]T and d-$(T\underline{p})_{8}[^{3}H]T$ are taken up at approximately the same rates and to the same extents which suggests there is no size restriction to uptake over this chain-length range.

Examination of lysates of cells exposed to the labeled methylphosphonates for 18 hrs. showed ∿70% of the labeled thymidine was associated with intact oligomer while the remainder was found in thym dine triphosphate and in cellular DNA. These results suggest the methylphosphonates which are recovered intact from the culture medium are slowly degraded within the cell. This degradation may result fror cleavage of the 3'-terminal [^{3}H]thymidine N-glycosyl bond with subsequent reutilization of the thymine base. The relatively l ng half li of the oligodeoxyribonucleoside methylphosphonates may be of value in potential pharmacological applications of the analogs.

Uptake experiments with <u>E.coli</u> B cells show they are permeable t d-A<u>p</u>[^{3}H]T, d-T<u>p</u>[^{3}H]T, and d-T<u>pT</u><u>p</u>[^{3}H]T, but not to d-$(T\underline{p})_{4}[^{3}H]T$ or d-$(T\underline{p})_{8}[^{3}H]T$. Thus, it appears analogs longer than 4 nucleotide unit cannot enter the bacterial cell. This size cutoff agrees with that found by others for oligosaccharides and oligopeptides (27,28). Similar results were obtained for other wild type gram positive and g negative bacteria such as <u>Bacillus subtilis</u> and <u>Pseudomonas auerogenc</u> Oligomers up to 7 nucleotides in length (e.g. d-A<u>p</u>G<u>p</u>G<u>p</u>A<u>p</u>G<u>p</u>G<u>p</u>[H]T) ar taken up by a permeable mutant of <u>E.coli</u>, <u>E.coli</u> ML 308-225. The out membrane of the cell wall of this mutant contains only small quantiti f lip polysaccharide (29) which may increase the permeability of the cell wall toward the l nger oligonucleoside methylph sph nates.

CELLULAR PROTEIN SYNTHESIS AND GROWTH

Nonionic oligonucleotides which inhibit cell free aminoacylation of tRNA or cell free protein synthesis also inhibit cellular protein synthesis and growth of bacterial cells and transformed hamster and human cells in culture (17,21,26). For example, $G^{m}_{p}(Et)G^{m}_{p}(Et)U$ inhibits cellular protein synthesis in a dose-dependent manner in transformed Syrian hamster fibroblasts (up to 90% at 100 μM). During prolonged incubation, protein synthesis is inhibited for the first 4 hrs. and then resumes at approximately the same time when oligomer uptake begins to level off. Cellular RNA synthesis, however, increases slightly during the first 4 hrs. and then returns to control levels. The reversible inhibitory effects most likely occur as a result of degradation of the triester within the cell.

As shown in Table VII, $G^{m}_{p}(Et)G^{m}_{p}(Et)U$ and oligonucleoside methylphosphonates which inhibit cell-free aminoacylation and protein synthesis also inhibit growth of mammalian and bacterial cells as assayed by their effects on colony formation.

TABLE VII. Effects of Nonionic Oligonucleotides on Colony Formation by Bacterial and Mammalian Cells in Culture

	% Inhibition			
	E. Coli B		BP-6[a]	HTB1080[b]
Oligomer	50 μM	160 μM	50 μM	50 μM
d-ApApA	3	44	29	31
d-ApApApA	19	78	36	19
d-GpGpT	7	11	7	9
$G^{m}_{p}(Et)G^{m}_{p}(Et)U$	-	-	50[c]	-

(a) BP-6 = transformed Syrian hamster fibroblasts.
(b) HTB1080 = Human tumor cells.
(c) [oligomer] = 25 μM.

This inhibition may occur as a result of binding of the analogs to complementary sequences on cellular tRNAs and mRNAs. The triester, $G^{m}_{p}(Et)G^{m}_{p}(Et)U$, was found t be a more effective inhibit r of BP6 c lony f rmation than was d-GpGpT. This result is consistent with the relative inhibitory effects of these ligomers n cell-free aminoacylati n (see Table IV).

Oligonucleoside methylphosphonates which are complementary to the Shine-Dalgarno sequence of 16S rRNA inhibit protein synthesis in E. coli ML 308-225 but not in E.coli B cells. Thus, for example, d-ApGpGpApGpGpT inhibits protein synthesis 20-45% but has no effect on RNA synthesis. This heptamer is taken up by E.coli ML 308-225 but n t E.coli B. d-ApGpGpT has no effect on either cellular protein or RNA synthesis. This lack of inhibition was also observed in cell-free systems (see Table VI). d-ApGpGpApGpGpT also specifically inhibits colony formation by E.coli ML 308-225 (see Table VIII). This analog and d-ApGpGpT had no effect on colony formation by E.coli B and only a small inhibitory effect on colony formation by transformed human cells.

TABLE VIII. Effects of Deoxyribonucleoside Methylphosphonates on Colony Formation by Bacterial and Human Cells

Oligomer	% Inhibition		
	E. coli B[b]	E.coli ML 308-225[b]	HTB1080[c]
d-GpGpT	-	5	-
d-ApGpGpT	0	0	-
d-ApGpGpApGpGp	0	78 - 97	-
d-ApGpGpApGpGpT	0	67 - 97	10

(a) [oligomer] = 75 μM.
(b) At either 22°C or 37°C.
(c) HTB1080 = Human tumor cells at 37°C.

We have also begun to investigate the possibility of inhibiting processing (splicing) of pre-mRNA by oligonucleoside methylphosphonates. For example, we have prepared analogs complementary to nucleotides 5 through 10 (d-GpGpTpApApG) and 8 through 13 (d-CpCpApGpGpTp) of U_1 RNA. These sequences encompass the region of U_1 RNA believed to be involved in pre-mRNA splicing (30,31). We have also prepared a nonamer, d-ApApTpApCpCpTpCpA, which is complementary to the exon/intr n junction of the donor splice site of SV40-large T-antigen pre-mRNA.

As shown in Table IX, d-CpCpApGpGpTp inhibits the growth of transformed human fibroblasts in mass culture and also inhibits c lony formation by transformed Syrian hamster fibroblasts. The greater inhibition of colony formation by the hexamer may result from perturbation of the cells during the critical period of attachment of the cells to the dish. The effects of d-CpCpApGpGpTp on hamster cell protein synthesis and RNA synthesis were also examined. In these experiments, RNA synthesis was inhibited 66% in the presence f 50 μM oligomer, while protein synthesis was inhibited 25%.

TABLE IX. Effects of d-CpCpApGpGpTp on Growth and Colony Formation by Mammalian Cells in Culture

Oligomer Conc. (μM)	% Inhibition HTB1080[a] Growth	% Inhibition BP6[b] Colony Formation
25	-	47
50	5	78
75	-	94
100	30	-
200	53	-

(a) HTB1080 = Transformed human fibroblasts.
(b) BP6 = Transformed Syrian hamster fibroblasts.

The effects of d-ApApTpApCpCpTpCpA, d-GpGpTpApApG and d-$(Tp)_5T$ on T-antigen synthesis in SV40-infected African green monkey kidney cells (BSC40) were studied. None of these oligomers (25 μM) show any cytotoxic effects on the growth of the BSC40 cells over a three-day period. The production of T-antigen was determined by an immunofluorescent assay after BSC40 cells were infected with SV40 in the presence of oligomer for 27 hours. Table X shows both d-ApApTpApCpCpTpCpA and d-GpGpTpApApG lower the levels of T-antigen in the infected cells sufficiently to prevent its detection by the antibodies. d-$(TP)_5T$ on the other hand appears to have little or no effect.

TABLE X. Effects of Oligonucleoside Methylphosphonates on SV40-Infected African Green Monkey Kidney Cells

Oligomer	Conc. μM	% Reduction of T-antigen Positive Nuclei
d-ApApTpApCpCpTpCpA	1	20
	5	30
	25	45
d-GpGpTpApApGp	1	10
	5	25
	25	30
d-$(Tp)_5T$	1	6
	5	6
	25	0

The results of our experiments suggest mRNA function may be selectively inhibited by nonionic oligonucleotides at two levels. Oligomers may be designed to inhibit translation of mRNA or alternatively processing of pre-mRNA may be prevented. In theory it should be possible to specifically inhibit the function of a single cellular r viral mRNA. Experiments are underway in our laboratory to further characterize and extend selective inhibition of nucleic acid function by oligonucleoside methylphosphonates.

ACKNOWLEDGEMENT

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APPENDIX B

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Effect of Deoxynucleoside Phosphorothioates Incorporated in DNA on Cleavage by Restriction Enzymes*

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DNA synthesized *in vitro* using deoxynucleoside phosphorothioates as substrates is quite similar to normal DNA in its biochemical properties (Vosberg, H.P., and Eckstein, F. (1977) *Biochemistry* 16, 3633-3640). In order to investigate the effect of phosphorothioate groups in DNA on the cleavage pattern of restriction endonucleases phosphorothioate double-stranded, circular, replicative form of fd DNA was synthesized *in vitro* with *Escherichia coli* DNA polymerase I using native single-stranded DNA as template and mixtures of three normal nucleotides and one nucleoside phosphorothioate analogue as substrates. The double-stranded products were hybrids with respect to their phosphorothioate content. Restriction analysis of normal and phosphorothioate DNA with the restriction endonucleases *Hae* III, *Bam* HI, *Hpa* II, *Hin*dII, *Alu* I, and *Taq* I showed that the enzymes were inhibited to different degrees depending on which of the nucleotides was replaced by the phosphorothioate. Most significant, inhibition was seen throughout with those DNAs which contained a phosphorothioate exactly at the cleavage site. Phosphorothioate substitutions at other positions, but still within the recognition sequences, were, except for *Alu* I, not or weakly inhibitory. Phosphorotioate nucleotides not present in the recognition sequences, did not affect at all the fragment patterns. The results show that recognition sequences of restriction endonucleases can be selectively protected against cleavage by base-specific introduction of phosphorothioate groups into DNA.

The present study on the influence of phosphorothioate groups in DNA on the activity of restriction enzymes arose from previous results demonstrating essentially normal incorporation of deoxynucleoside phosphorothioates into DNA (1). These nucleotides carry a sulfur instead of an oxygen on the phosphorus of the deoxynucleoside triphosphate. Synthesis in the previous study was carried out with the single-stranded DNAs of the phages φX174 and fd as templates in cell-free extracts of *Escherichia coli* or with *E. coli* DNA polymerase I. The products were double-stranded RF[1] DNA consisting of one native unmodified strand and a complementary strand in which one of the normal nucleotides was replaced by th corresponding nucleoside phosphorothioate. Not only synth - sis but also ring closure of nicked double-stranded circular DNA molecules occurred readily. Product analysis of phosphorothioate phage DNAs did not exhibit gross changes in the physiochemical properties of these DNAs as compared to their unmodified counterparts.

Because of the known slow enzymatic hydrolysis of phosphorothioate internucleotidic linkages by snake venom phosphodiesterase (2) and the nucleases associated with *E. coli* DNA polymerase I (2, 3) as well as T_4 DNA polymerase (4), we decided to investigate the restriction cleavage pattern of phosphorothioate fd DNAs which had been synthesized in the presence of one of the four nucleotide analogues. With each of six restriction endonucleases, the consequences of differential replacement of any one of the four normal nucleotides by th respective phosphorothioate analogue were investigated. Four of the enzymes tested cut DNA frequently (*Hae* III, *Hpa* II, *Alu* I, *Taq* I), whereas two cut infrequently (*Bam* HI, *Hin*dII). Our results show that, in general, a phosphorothioate group present at the site of cleavage decreases the rate of hydrolysis.

MATERIALS AND METHODS

Nucleotides—Deoxyadenosine, deoxycytidine, deoxyguanosine, and deoxythymidine 5′-*O*-(1-thiotriphosphate) were prepared as published for ATPαS (5). The chemically synthesized mixtures of diastereomers of these nucleotides were used. [^{3}H]dTTP was purchased from Amersham/Buchler with a specific activity of 19.6 Ci/mmol. Nonradioactive nucleotides were obtained from Boehringer Mannheim.

Enzymes—Proteinase K (EC 3.4.21.14) was from Boehringer Mannheim. *E. coli* DNA polymerase I (EC 2.7.7.7) with an activity of ~10,000 units/ml and a specific activity of 2500 units/mg was kindly provided by Dr. K. Geider, Heidelberg. T_4 DNA ligase (EC 6.5.1.1) was purified essentially according to a published procedure (6) from *E. coli* B infected with T_4 amN82 (7). Restriction endonuclease *Alu* I (EC 3.1.23.1) was a gift from Dr. T. Meyer, Heidelberg. The restriction endonuclease *Hae* III (EC 3.1.23.17) was isolated according to Roberts *et al.* (8) by Dr. T. Winkler, Heidelberg. Endonucleases, *Hin*dII (EC 3.1.23.20), *Bam* HI (EC 3.1.23.6), *Taq* I (EC 3.1.23.39), and *Hpa* II (EC 3.1.23.24) were from Boehringer Mannheim.

Other Materials—Agarose (L.E. grade) was from M. C. I. Biomedical, Rockland, ME. Ethidium bromide was from Boehringer Mannheim.

Synthesis of fd RF in Vitro—Wild type single-stranded fd DNA was extracted from phage particles as described (9). The reaction conditions were essentially according to Oertel and Schaller (10). The assay volumes (2 ml) contained 200 μg of single-stranded fd DNA in 50 mM Tris-HCl, pH 8.1, 0.1 M KCl, 6 mM $MgCl_2$, 50 μg/ml bovine serum albumin. Synthesis was primed with oligonucleotides (approx-

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[1] The abbreviations used are: RF, double-stranded, circular, replicative form of fd DNA; dNMP, the four common 2′-deoxynucleoside monophosphates; dNMPS, 2′-deoxynucleoside 5′-*O*-phosphorothioate; dNMPαS, 2′-deoxynucleoside 5′-*O*-(1-thiotriphosphate); dNMPS-RF, RF DNA containing dNMPS; RFI, circular, covalently closed supercoiled fd DNA; RFII, double-stranded, circular fd DNA with a discontinuity in at least one strand; RFIII, double-stranded, linear fd DNA; RFIV, circular, covalently closed relaxed fd DNA.

EXHIBIT B

imately 15 to 20 bases long) prepared from denatured calf thymus DNA by limited digestion with pancreatic DNase and subsequent fractionation of fragments on Sephadex G-100. Four to six primer molecules were present per fd DNA molecule on the average. Normal nucleotides were 0.5 mM each. Deoxynucleoside phosphorothioates were 1 mM. To monitor fd RF synthesis, [^{3}H]dTTP (specific activity, 240 mCi/mmol) or [^{3}H]dATP (specific activity, 180 mCi/mmol) was included in the mixture.

Approximately 100 units of DNA polymerase I were applied per assay. Prior to addition of the enzyme, the reaction mixture was preincubated for 15 min at 45 °C in order to anneal the priming oligonucleotides to the fd DNA templates. Synthesis was carried out overnight at room temperature. After approximately 12 to 15 h, ATP was added (0.5 mM final concentration) together with roughly 10 units of T_4 DNA ligase. The mixture was then incubated for 3 h at 30 °C and the reaction was stopped by addition of 10 mM EDTA. To remove free nucleotides, DNA was passed over a Bio-Gel A-1.5m column (38 × 1.5 cm) and eluted with 10 mM Tris-HCl, pH 7.6, 20 mM NaCl, 1 mM EDTA. The peak fractions containing fd RF were collected and centrifuged in CsCl density gradients containing ethidium bromide to separate covalently closed RFIV from nicked and linear RF molecules (11). The lower band DNA was cut out from the gradient and, after butanol extraction of the dye, dialyzed against Tris-HCl, pH 7.6, 20 mM NaCl, 1 mM EDTA. About 40% of the input single-stranded fd DNA was usually converted into RFIV DNA.

Restriction Enzyme Assays—Incubation mixtures contained the following buffers. *Bam* HI and *Hae* III: 6 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol; *Hpa* II: 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 6 mM KCl, 1 mM dithiothreitol; *Hin*dII: 10 mM Tris-HCl, pH 7.9, 7 mM $MgCl_2$, 60 mM NaCl, 6 mM β-mercaptoethanol; *Alu* I: 6 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol; *Taq* I: 10 mM Tris-HCl, pH 8.4, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol. All reaction volumes were 30 μl. *Taq* I assays were incubated at 65 °C, all others at 37 °C.

DNA Fragment Analysis—The restriction fragments were analyzed by gel electrophoresis in either 1% agarose (in 40 mM Tris-acetate, pH 7.8, 5 mM sodium acetate, 0.5 mM EDTA) or 10% polyacrylamide (in 45 mM Tris-borate, pH 8.3, 1.4 mM EDTA). DNA was visualized after staining with ethidium bromide with short wavelength ultraviolet light (286 nm) and photographed with Agfapan Type 100 professional film.

RESULTS

Restriction by Hae III Endonuclease—This enzyme recognizes the nucleotide sequence 5′-GGCC-3′ and produces blunt ended polynucleotides by cutting between G and C. Ten fragments are generated with normal fd RF (12). Phosphorothioate fd RF shows an altered pattern of restriction fragments only after replacement of dCTP by dCTPαS. Substitution of other nucleotides does not change the distribution of fragments as compared to cleavage of normal DNA (Fig. 1). A cl se inspection of the bands produced with dCMPS-RF exhibits roughly two classes of fragments: a minor fraction of fragments, most of them smaller than the largest normal fragment A (2528 base pairs) (12), and second, a major fraction of fragments all larger than fragment A. On 1% agarose gels which resolve these larger fragments better than do 10% polyacrylamide gels, at least four discrete bands are discriminated in this region (not shown). It can be deduced from the known *Hae* III cleavage map of fd RF that these fragments arise from infrequent cuts randomly occurring at some of the *Hae* III sites of this DNA. The length distribution of these intermediates suggests that probably not more than 3 out of 10 possible cuts occurred in most of these DNA molecules, leading to larg fragments with overlapping sequence organization. We assume, theref re, that *Hae* III cleavage sequences ar more or less equally affected by the phosphorothioate substitutions with dCTPαS.

The experiment in Fig. 1 was done with 1 unit of enzyme/DNA sample. Increasing the concentration up to 10 units/sample was not sufficient to produce the normal limit products with dCMPS-RF. Even at this high enzyme concentration, most of the fragments were found at intermediate positions,

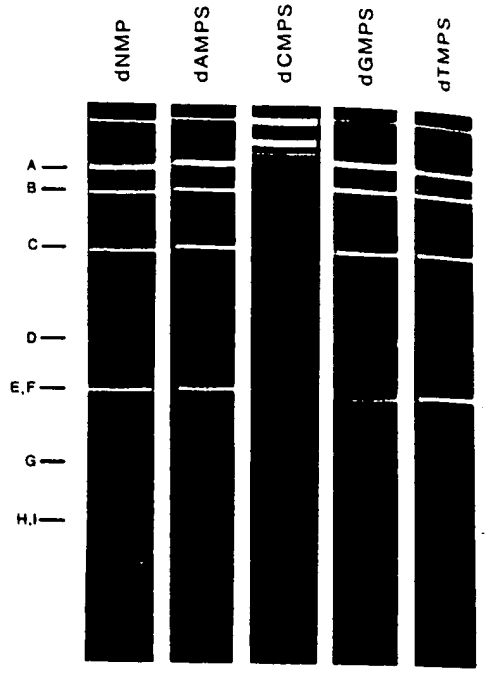

FIG. 1. **Restriction by *Hae* III endonuclease.** 1-μg sample normal or phosphorothioate fd RF-DNA were incubated for 30 m with approximately 1 unit of enzyme. Fragments were analyzed on 10% polyacrylamide gel. Positions of *fragments A* to *I* of normal RF are indicated. Fragment J is not resolved by the ethidium-staining procedure. The substituting nucleotides are depicted on *top* of the corresponding *lanes*. The *left lane* is designated by dNMP and contains unsubstituted fd RF as a control.

indicating a strong inhibitory effect of dCMPS on the *Hae* III activity. Although we cannot exclude the possibility that some of the recognition sequences are totally refractive to cleavage due to mismatching, we take these results as indication that the primary cause for cleavage inhibition is the presence of two phosphorothioate groups adjacent to the C residues in the cleavage sequence, with one being at the cleavage site itself. If mismatching were a frequent event, a substitution of dGTP by dGTPαS should also produce a significant inhibition of cleavage by this enzyme, which is not observed.

Restriction by Bam HI Endonuclease—This enzyme recognizes the sequence 5′-GGATCC-3′ and cleaves between the two G residues. Normal fd RF has two cleavage sites for *Bam* HI (12). The most prominent inhibition is seen after replacement of dGTP by dGTPαS (Fig. 2). dCTPαS inhibits to a lesser degree. Neither of the other two nucleotide analogues exerts any effect on the cleavage activity of this enzyme. A 3-fold increase of the enzyme concentration resulted in complete digestion even of dGMPS-RF. Similar to *Hae* III, the most conspicuous effect on *Bam* HI activity is seen with that nucleoside phosphorothioate which is incorporated into the cleavage site of this enzyme.

Restriction by Hpa II Endonuclease—This enzyme which recognizes the sequence 5′-CCGG-3′ generates 15 fragments with normal fd RF (12). Most prominent inhibition of *Hpa* II is observed after replacement of dCTP by dCTPαS (Fig. 3). Very little inhibition is seen in DNA containing dGMPS, but the other two nucleotide analogues do not alter the cleavage pattern. An increase of th enzyme concentration up to 5-fold has only a moderate effect on the length distribution of fragments obtained with dCPMS-RF (not shown). Thus, the inhibition of this enzyme by the presence of phosphorothioate groups in fd DNA is str ng.

Restriction by Alu I Endonuclease—The cleavage sequence of this enzyme is 5′-AGCT-3′. fd RF is normally cut into 16

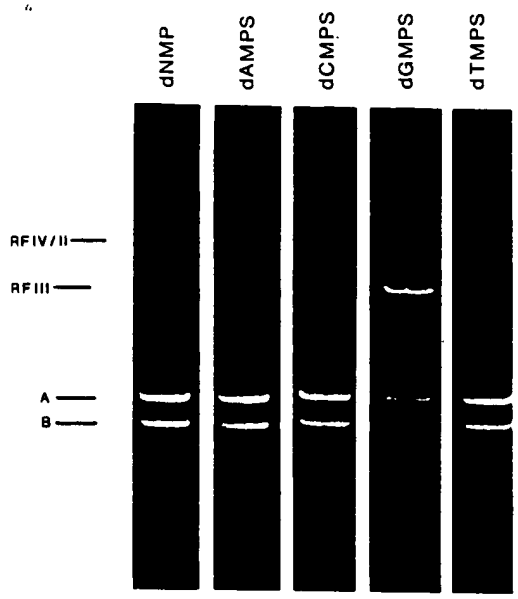

FIG. 2. **Restriction by *Bam* HI endonuclease.** Mixtures containing 0.2 μg of normal or phosphorothioate fd RF were incubated with about 0.2 unit of enzyme for 30 min. Cleavage products were analyzed on a 1% agarose gel. *A* and *B* are the two standard fragments obtained with fd RF. Migration positions of RFIV (or RFII) and partially cleaved full length linears (RFIII) are marked. The substituting nucleotides are depicted on *top* of the corresponding *lanes*. The *left lane* is designated by dNMP and contains unsubstituted fd RF as a control.

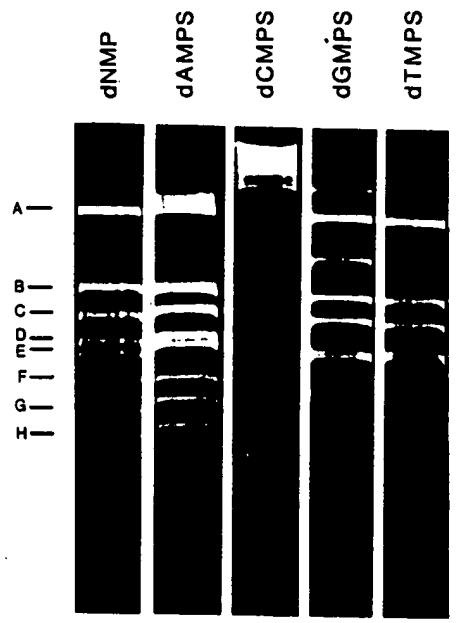

FIG. 3. **Restriction by *Hpa* II endonuclease.** The assays contained 1 μg of normal or phosphorothioate DNA and 1 to 2 units of enzyme. Incubation was for 30 min. Fragments were analyzed on a 15% polyacrylamide gel. The normal *fragments A* to *H* are visible; the smaller fragments I to 0 are not resolved. The substituting nucleotides are depicted on *top* of the corresponding *lanes*. The *left lane* is designated by dNMP and contains unsubstituted fd RF as a control.

fragments (12). In contrast to the other enzymes tested, *Alu* I is inhibited by all four nucleotide substituents. There is, however, a gradient in the degree of inhibition by the individual analogues. Inhibition is strongest by replacement of dCTP by dCTPαS. Since cleavage occurs between G and C, substitution of the latter is accompanied by placing a phosphorothioate group at the cleavage site. From the size distributions observed after cleavage of the different phosphorothioate DNAs, we deduce the following order of inhibitory strength: dCTPαS > dTTPαS > dATPαS ≫ dGTPαS.

Fig. 4 shows the cleavage products of dAMPS-RF and of dCMPS-RF after electrophoresis on 1% agarose. The complex fragment distributions of the two different phosphorothioate DNAs were consistently reproduced. The gel patterns show little overlapping in the composition of the fragments produced with *Alu* I. At least 10 bands obtained with dCMPS-RF are not seen after cleavage of dAMPS-RF, and 6 fragments produced with dAMPS-RF are not detected among the dCMPS-RF bands. The distribution of fragments after cleavage of dTMPS-RF (not shown here) does not coincide with either of the sets of fragments in Fig. 4. (dGMPS-RF is only weakly inhibitory. *Alu* I cleavage leads to a mixture of normal limit products and only some extra fragments not seen with unsubstituted DNA.) These results indicate that different substitutions within a given *Alu* I recognition sequence have differential effects on the cleavability of this sequence. The reason for this complex behavior of *Alu* I is not known. Conceivably, this enzyme recognizes more than only the tetranucleotide AGCT.

Digestion with higher concentrations of enzyme as, *e.g.* applied in the experiments of Fig. 4 suggests strong inhibition of *Alu* I by dATPαS, dCTPαS, and dTTPαS, respectively, and weak inhibition by dGTPαS.

Restriction by HindII Endonuclease—This enzyme recognizes the general sequences 5′-GTPYPUAC-3′ and cleaves

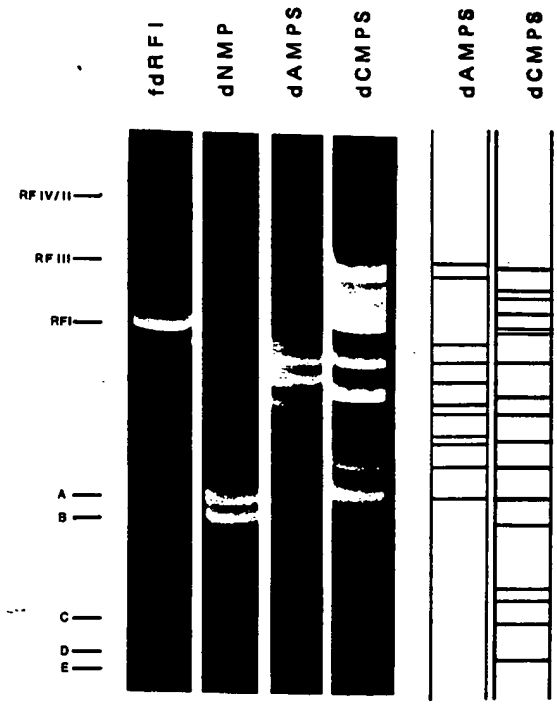

FIG. 4. **Restriction by *Alu* I endonuclease.** Three mixtures contained 1 μg of normal fd RF, dAMPS-RF, and dCMPS-RF, respectively. Normal RF was incubated with approximately 1 unit of enzyme for 30 min. dAMPS-RF and dCMPS-RF were incubated for 90 min with 5 to 10 units of enzyme each. The cleavage products were analyzed on a 1% agarose gel. The *left lane* contains untreated fd RFI as a position marker. The lanes with the cleavage products are marked by dNMP (normal DNA), dAMPS (dAMPS-RF), and dCMPS (dCMPS-RF). Positions of the normal *fragments A* to *E* are designated on the left side. Fragments F to P were already eluted from the gel. The schematic presentation of cleavage fragments on the *right side* demonstrates numbers and relative positions of bands obtained with dAMPS-RF and dCMPS-RF, respectively. These patterns were derived from a densitometric scan of the Agfapan film used for photography of the gel.

between the central pyrimidine and purine residues. This sequence occurs in normal fd RF once with T as pyrimidine and A as purine, respectively (12). Inhibition of this enzyme is seen only after substitution of dATP by dATPαS (Fig. 5). The inhibitory effect of this analogue is moderate. A 5-fold increase in enzyme concentration is sufficient for complete cleavage of dAMPS-RF.

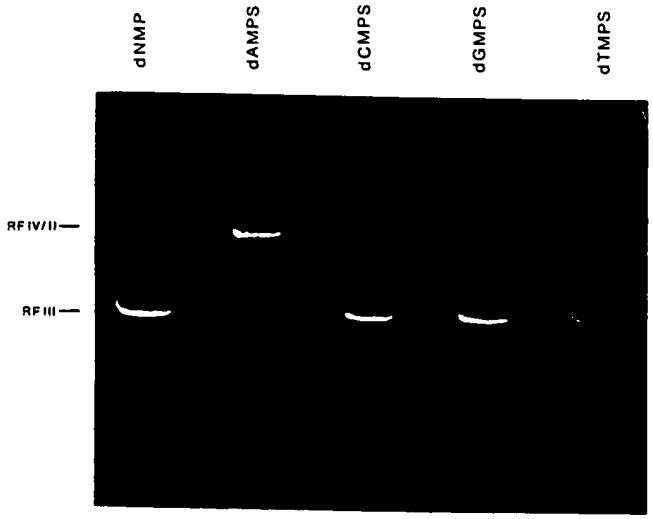

FIG. 5. **Restriction by *HindII* endonuclease.** The assays contained 0.1 μg of normal or phosphorothioate fd RF. The DNAs were digested with approximately 0.1 unit of enzyme for 30 min. Reaction products were analyzed on a 1% agarose gel. The substituting nucleotides are indicated on *top* of the corresponding *lanes*. The *left lane* (designated by dNMP) contains unsubstituted fd RF.

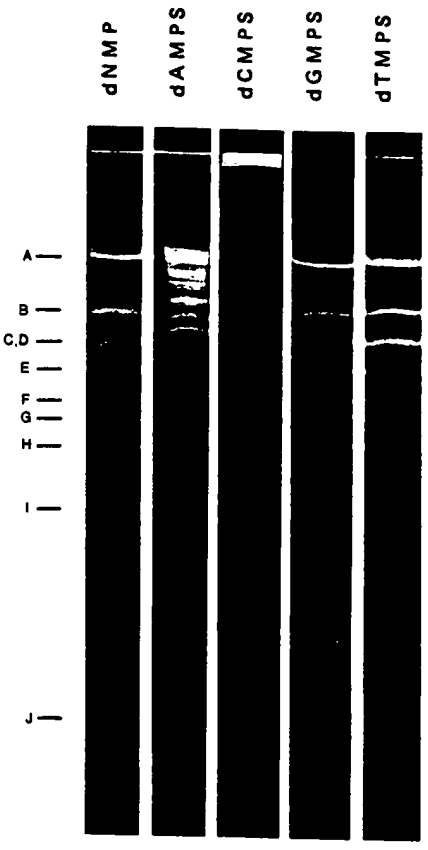

FIG. 6. **Restriction by *Taq* I endonuclease.** The assays contained 1 μg of normal or phosphorothioate fd RF and about 1 unit of enzyme each. Samples were incubated for 30 min. Products were analyzed on a 10% polyacrylamide gel. Positions of normal *fragments* *A* to *J* are indicated. The substituting nucleotides are designated on *top* of the corresponding *lanes*. The *left lane* (dNMP) contains unsubstituted fd RF.

Restriction by Taq I Endonuclease—Taq I is active at 65 °C and cleaves within the recognition sequence 5'-TCGA-3' between T and C. It produces 10 fragments with normal fd RF DNA (12). The most striking effect on cleavage is seen with dCTPαS substituting for dCTP (Fig. 6). Inhibition is moderate. A 5-fold increase in enzyme concentration was sufficient to produce an almost normal length distribution of fragments. dATPαS has a slight effect on restriction. However, dGTPαS and dTTPαS both do not affect the activity of this enzyme at all. Again, a phosphorothioate group at the cleavage site is significantly more operative than substitutions at other locations.

DISCUSSION

Nucleoside phosphorothioates have found wide application for the determination of the stereochemical course of enzymatic nucleotidyl and phosphoryl transfer reactions (13–15). However, the increased stability of these compounds against enzymatic hydrolysis has also made some of them useful for an understanding of the role played by the parent compounds in some more complex biochemical systems (13).

We had earlier incorporated dNTPαS into fd and φX174 DNA, mainly with the hope that the sulfur might react with heavy metals as a first step in the development of a physical method to sequence DNA (1). Although complexes of platinum reacted stoichiometrically with the phosphorothioate analogues of poly(A-U) (16) and tRNA (17), the reaction with phosphorothioate DNA has so far been unsatisfactory.

Our previous results indicated (i) a rate of incorporation of these nucleotide analogues into fd or φX174 RF DNA which was only moderately below the rate obtained with normal nucleotides, and (ii) the absence of gross changes in a variety of biochemical properties of phosphorothioate DNA (1). Further, we deduced from the amount of [^{35}S]dATPαS incorporated into φX174 RF that the base composition of phosphorothioate DNA was very close to that of normal DNA. Independent data supporting this view have recently been presented by Kunkel *et al.* (4). There, *in vitro* synthesis of phosphorothioate phage DNA was combined with *in vivo* analysis of reversion frequencies. Calculated from the observed reversion rates of normal and phosphorothioate DNA synthesized with *E. coli* DNA polymerase I, an increase in the error frequency by a factor of 20 was calculated. This increase is due to a selective inhibition of the 3′ → 5′ proofreading exonucleolytic activity of this DNA polymerase. Thus, with phosphorothioate nucleotides, the rate of errors in DNA increases from 2×10^{-6} to about 1×10^{-5}.

Since phosphorothioate diesters are also hydrolyzed much more slowly than phosphate diesters by other phosphodiesterases such as snake venom phosphodiesterase (2), we decided to compare restriction patterns obtained with normal and phosphorothioate fd RF DNA to see whether these enzymes too were slow in hydrolyzing phosphorothioate diesters.

Of the six restriction enzymes tested, all exhibited distinctly altered patterns of restriction fragment formation with phosphorothioate DNA (see Table I). The most significant inhibitory effects were seen with those phosphorothioate DNAs expected to contain a nucleoside phosphorothioate within the otherwise correct recognition sequence at the site of cleavage. The enzyme *Hae* III, for example, cuts between G and C within the sequence 5′-GGCC-3′. DNA synthesis in the presence of dCTPαS should place a phosphorothioate group on the 5′ side of C and on the 3′ side of that G flanking the cleavage site. We observe that, whatever the actual locations of phosphorothioate groups are, fd RF synthesized with

TABLE I

Inhibition of restriction endonucleases by phosphorothioate deoxynucleosides

Enzyme	Recognition sequence[a]	Inhibiting nucleotide	Strength of inhibition
Hae III	↓ GGCC	dCTPαS	Strong
Bam HI	↓ GGATCC	dGTPαS	Moderate
Hpa II	↓ CCGG	dCTPαS	Strong
		dGTPαS	Weak
Alu I	↓ AGCT	All four	Strong with dCTPαS, dTTPαS, and dATPαS, weak with dGTPαS
*Hin*dII	↓ GTTAAG[b]	dATPαS	Strong
Taq I	↓ TCGA	dCTPαS	Moderate
		dATPαS	Weak

[a] Cleavage sites are indicated by arrows.
[b] The general sequence is GTPYPUAG.

dCTPαS is cleaved by *Hae* III at a significantly reduced rate. A common feature shared by all six enzymes tested is that inhibition is strongest with those DNAs which contain a phosphorothioate diester at the cleavage site. This result is summarized in Table I. Thus, *Bam* HI is inhibited by dGMPS residues in the DNA, *Hin*dII by dAMPS, and *Alu* I, *Hpa* II, *Hae* III and *Taq* I by dCMPS. To investigate the specificity of this inhibition with respect to the position of the phosphorothioate group, it was important to assess the influence of phosphorothioate substitutions at other positions in the recognition sequence. If there was an influence on the rate of cleavage by phosphorothioate groups at other than the cleavage site, one would expect also to see inhibition by such substitutions. These experiments show, however, that dGMPS-DNA is readily cleaved by *Hae* III. DNA synthesized in the presence of dATPαS, dCTPαS, and dTTPαS is an almost normal substrate for *Bam* HI and the presence of dGMPS or dTMPS does not alter the substrate properties of such DNA for *Hin*dII. However, there is some inhibition of *Hpa* II by dGMPS incorporation and *Alu* I is not only inhibited by dCTPαS as expected but also by the other three analogues to various degrees in the approximate order dTTPαS > dATPαS ≫ dGTPαS. Nucleotide analogues which do not normally occur within the recognition sequence such as dATPαS in the *Hae* III sequence have no effect on the cleavage pattern (see data for *Hae* III, *Hpa* II, and *Hin*dIII in Table I).

Thus, the interpretation of the cleavage patterns is straightforward for the enzymes *Hae* III, *Hin*dII, and essentially also for *Bam* III. The conclusion there is that the inhibition observed is due to the incorporation of a phosphorothioate at the cleavage site. That this inhibition is due to the slow hydrolysis of the phosphorothioate diester rather than a mismatch at the cleavage site introduced during polymerization in the presence of the phosphorothioates is evident from the data provided by Kunkel *et al.* (4) on the error frequency of *E. coli* DNA polymerase I. The factor of 20 for the increase in errors when deoxynucleoside phosphorothioates are substrates, although significant in terms of mutational stability of DNA, is too small to be resolved by our restriction pattern analysis.

Inhibition of the enzymes *Hpa* II, *Alu* I, and *Taq* I is also observed when the other nucleotides present in the recognition sequence are replaced by phosphorothioates although inhibition by introduction of a phosphorothioate into the cleavage site is strongest. Since the weaker inhibition of these enzymes observed with some other phosphorothioate nucleotides cannot be due to base mismatching (4), it probably has its cause in some as yet unidentified influence of the neighboring groups on the rate of hydrolysis at the cleavage site.

The results presented here imply the practical consequence of selective protection of *in vitro*-made DNA against unwanted degradation by restriction endonucleases. For instance, cDNA could be synthesized in the presence of one of the deoxynucleoside phosphorothioates. Subsequent j ining of this DNA to appropriate DNA linkers containing defined recognition sequences for restriction enzymes would allow processing of the linker regions with the cDNA fragment being insensitive to the processing restriction endonuclease. Similarly, by incorporation of dNTPαS at sticky ends of restriction endonuclease fragments, these can be protected against degradation by exonucleases (18). Thus, these analogues may be useful for certain *in vitro* manipulations of DNA.

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APPENDIX C

Synthesis and Characterization of an Octanucleotide Containing the *Eco*RI Recognition Sequence with a Phosphorothioate Group at the Cleavage Site†

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ABSTRACT: The synthesis and characterization of an octanucleotide, d(GGsAATTCC), containing the recognition sequence of the *Eco*RI restriction endonuclease with a phosphorothioate internucleotidic linkage at the cleavage site are described. Two approaches for the synthesis of the R_P and S_P diastereomers of this octamer by the phosphite method are presented. The first consists of the addition of sulfur instead of H_2O to the phosphite at the appropriate position during chain elongation. This method results in a mixture of diastereomers that can be separated by high-performance liquid chromatography after 5′-terminal phosphorylation. The second uses the presynthesized and diastereomerically pure dinucleoside phosphorothioate d[Gp(S)A] for the additi n to the growing oligonucleotide chain as a block. The products are characterized by digestion with nuclease P1, fast atom bombardment mass spectrometry, ^{31}P NMR spectroscopy, and conversion to d(GGAATTCC) by desulfurization with iodine. Only the R_P diastereomers of d(GGsAATTCC) and its 5′-phosphorylated derivative are cleaved by *Eco*RI endonuclease. The rate of hydrolysis is slower than that of the unmodified octamer. The phosphorothioate octamer will be useful for the determination of the stereochemical course of the *Eco*RI-catalyzed reaction.

Restriction endonucleases catalyze the cleavage of double-stranded DNA at sequence-specific sites. Although these enzymes are immensely important in genetic engineering, little mechanistic information is available [see review by Modrich (1982)]. The recent advances in the efficient synthesis of small oligonucleotides have made it possible to undertake a variety of mechanistic investigations with these enzymes. We had observed earlier (Vosberg & Eckstein, 1982) that certain restriction enzymes including *Eco*RI (B. V. L. Potter, H. P. Vosberg, and F. Eckstein, unpublished results) are capable of cleaving phosphorothioate internucleotidic linkages when incorporated into the (−) strand of fd DNA, although at reduced rates. This suggested to us that it should be feasible to determine the stereochemical coursé of such an enzyme reaction providing we could synthesize an oligonucleotide containing the appropriate recognition sequence with a phosphorothioate internucleotidic linkage of known absolute configuration at the cleavage site. Endonuclease-catalyzed hydrolysis in the presence of $H_2{}^{18}O$ and subsequent nuclease P1 cleavage of the reaction products should furnish a deoxynucleoside 5′-[^{18}O]-phosphorothioate whose absolute configuration should be amenable to stereochemical analysis [see review by Eckstein (1983a,b)]. The knowledge of whether such an enzymatic reaction proceeds with retention or inversion of configuration at phosphorus provides evidence for or against the existence of a covalent enzyme intermediate and thus limits the number of mechanisms that can be proposed for an enzymatic reaction. We wish to report here the successful synthesis and characterization of the octanucleotide d(GGsAATTCC), which contains the recognition sequence for the restriction endonuclease *Eco*RI and a phosphorothioate group at the cleavage site. The determination of the stereochemical course of the reaction catalyzed by this enzyme using this octamer will be reported at a later date.

Materials and Methods

Nucleosides were obtained from Pharma-Waldhof (Düsseldorf, West Germany). Benzoyl chloride, anisoyl chloride, and isopropionyl chloride were purchased from EGA Chemie (Steinheim, West Germany) and were redistilled before use. 1*H*-Tetrazole was a product of EGA Chemie and was purified by sublimation at 100 °C and 0.05 mmHg prior to use. Pyridine, 2,6-lutidine, and *N*-ethyldiisopropylamine were purchased from Merck (Darmstadt, West Germany) and were refluxed with and then distilled from calcium hydride and stored over 4-Å molecular sieves. Acetonitrile used in the solid-phase nucleotide synthesis was an HPLC grade reagent from J. T. Baker Chemicals (Deventer, Holland). It usually contains 0.01% water but was stored over 4-Å molecular sieves and otherwise used as supplied. THF[1] used in the solid-phase synthesis was Merck dried reagent (maximum H_2O content 0.01%) as was Me_2SO (maximum H_2O content 0.03%) used to prepare phosphorothioate-containing dimers. These solvents were also stored over 4-Å molecular sieves. All other solvents used in the preparation of oligonucleotides were p.a. grade and were stored over 4-Å molecular sieves. Nuclease P1 (200 units/mg) was obtained from Sigma (Munich, West Germany), and alkaline phosphatase (from calf intestine, 2500 units/mg, molecular biology grade) was purchased from Boehringer Mannheim (West Germany). Polynucl otide kinase (from T4 infected *E. coli*, 5 units/μL) was a product

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[1] Abbreviations: FAB, fast atom bombardment; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; THF, tetrahydrofuran; Me_2SO, dimethyl sulfoxide; TEAA, triethylammonium acetate; TEAB, triethylammonium bicarbonate; EDTA, ethylenediaminetetraacetic acid; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; (R_P)- and (S_P)-d[Gp(S)A], R_P and S_P diastereomers of 5′-*O*-(2′-deoxyadenosyl) 3′-*O*-(2′-deoxyguanosyl) phosphorothioate; dAMPS, 2′-deoxyadenosine 5′-*O*-phosphorothioate; (R_P)- and (S_P)-d(GGsAATTCC), the R_P and S_P diastereomers of the octamer d(GGAATTCC) containing a d[Gp(S)A] unit instead of d(GpA); d(pGGsAATTCC), the 5′-phosphorylated octamer; $DMTdG^{ib}p(S,OCH_3)dA^{bz}_{morp}$, 5′-*O*-[*N*⁶-benzoyl-3′-*O*-(morpholinomethoxyphosphino)-2′-deoxyadenosyl] 3′-*O*-[*N*²-isobutyryl-5′-*O*-(dimethoxytrityl)-2′-deoxyguanosyl] *O*-methyl phosphorothioate; $DMTdG^{ib}p(S,OCH_3)dA^{bz}_{OH}$, 5′-*O*-(*N*⁶-benzoyl-2′-deoxyadenosyl) 3′-*O*-[*N*²-isobutyryl-5′-*O*-(dimethoxytrityl)-2′-deoxyguanosyl] *O*-methyl phosphorothioate; d, dalton.

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EXHIBIT C

of P-L Biochemicals (St. Goar, West Germany). Tetrabutylammonium hydroxide for HPLC was obtained from Waters Associates (Milford, MA) under the name PIC Reagent A. KH_2PO_4 (Merck, p.a. grade) used for HPLC was further purified by passage over Chelex resin to remove UV-absorbing impurities (Karkas et al., 1981). All other reagents were of the best quality available and were used as received. TLC was performed with silica gel 60 F_{254} plates (Merck, Darmstadt, West Germany). *Eco*RI endonuclease was isolated from an *Eco*RI overproducing strain kindly provided by Dr. M. Zabeau (Heidelberg) and was purified by chromatography on phosphocellulose and DEAE-cellulose as described (Langowski et al., 1980).

Methoxydichlorophosphine was prepared as described (Martin & Pizzolato, 1950). Methoxymorpholinochlorophosphine was synthesized by the procedure of McBride & Caruthers (1983) using *N*-(trimethylsilyl)morpholine (Pike & Schauch, 1962) as starting material. 5′-*O*-(Dimethoxytrityl)thymidine, N^6-benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxyadenosine, and N^2-isobutyryl-5′-*O*-(dimethoxytrityl)-2′-deoxyguanosine were synthesized by the procedures of Schaller et al. (1963) as modified by Gait et al. (1982a). N^4-Anisoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidine was prepared by the method of Schaller et al. (1963). All these compounds were purified by flash chromatography (Still et al., 1978) on silica gel 60 (Merck, particle size 0.040–0.063 mm) using $CHCl_3$–CH_3OH mixtures under a positive nitrogen pressure of 0.5 atm. All dimethoxytrityl derivatives appeared pure by TLC using either $CHCl_3$–CH_3OH (95:5 v/v) or ethyl acetate–CH_3OH (95:5 v/v) as solvent. Protected deoxyribonucleoside morpholinomethoxyphosphites were synthesized by reacting the appropriate base-protected dimethoxytrityl nucleosides with methoxymorpholinochlorophosphine. The reaction conditions and purification protocols given by Dörper & Winnacker (1983) were followed with the exception that CH_2Cl_2 instead of $CHCl_3$ was used as the reaction solvent. After purification, the protected nucleoside methoxymorpholinophosphites appeared pure as judged by TLC using either $CHCl_3$–EtOAc–NEt_3 (45:45:10 v/v) or $CHCl_3$–CH_3OH–NEt_3 (85:5:10 v/v) and ^{31}P NMR spectroscopy (T derivative, δ 144.85 and 145.10; all other derivatives, δ 145.1 and 145.4 for the two diastereomers). Silica gel used as the solid support in oligonucleotide synthesis was Fractosil 200 (Merck, Darmstadt, West Germany) and was functionalized so as to possess free amino groups by the procedure of Caruthers (1982). N^4-Anisoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidine was attached via the 3′-hydroxyl group to this amino silica gel as reported by Caruthers (1982). A loading of 113 μmol of N^4-anisoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidine per gram of resin was achieved.

HPLC was performed with two Waters Associates Model 6000 A pumps controlled by a Model 660 solvent programmer. In all cases the reverse-phase octadecyl material ODS-Hypersil [5-μm particle size, supplied by Gynkotek (München, West Germany)] was utilized as the stationary phase although the buffers used for the mobile phase varied with the particular application. For the purification of dimethoxytrityl oligonucleotides, a linear gradient (flow rate 6 mL min^{-1}) consisting of 100 mM TEAA, pH 7 (A), and 100 mM TEAA, pH 7, containing 70% CH_3CN (B) was used (t = 0 min, 20% B; t = 20 min, 80% B) (gradient I). To purify completely deblocked ligonucleotides, a linear gradient (flow rate 3.5 mL min^{-1}) consisting of 100 mM TEAB, pH 8 (A), and 100 mM TEAB, pH 8, containing 60% CH_3CN (B) was used (t = 0 min, 5% B; t = 20 min, 30% B) (gradient II). This buffer system was further used, both to monitor the reactions and to purify the products of (1) the *Eco*RI-catalyzed hydrolysis of the various octanucleotides, (2) the desulfurization of phosphorothioate-containing oligomers with iodine, and (3) the 5′-phosphorylation of octanucleotides with polynucleotide kinase. The purity of the oligonucleotides was checked by using three systems. These all consisted of a 20-min linear gradient (flow rate 2 mL min^{-1}) produced from (1) 100 mM TEAA, pH 7.0 (A), and 100 mM TEAA, pH 7.0, containing 60% CH_3CN (B) (t = 0 min, 5% B; t = 20 min, 30% B) (gradient III), (2) 5 mM tetrabutylammonium hydroxide, pH 7.5, containing 4% CH_3CN (A) and 5 mM t trabutylammonium hydroxide, pH 7.5, containing 70% CH_3CN (B) (t = 0 min, 30% B; t = 20 min, 80% B) (gradient IV), and (3) 50 mM KH_2PO_4, pH 6 (A), and 50 mM KH_2PO_4, pH 6, containing 30% CH_3CN (B) (t = 0 min, 5% B; t = 20 min, 30% B) (gradient V). To resolve the nuclease P1 digestion products of the various oligonucleotides, an upward concave (Waters solvent programmer curve 9) gradient (flow rate 1 mL min^{-1}) prepared from 50 mM KH_2PO_4, pH 6.5 (A), and 50 mM KH_2PO_4, pH 6.5, containing 30% CH_3CN (B) was used (t = 0 min, 0% B; t = 15 min, 50% B) (gradient VI). To separate the nuclease P1–alkaline phosphatase codigestion products, a linear gradient (flow rate 2 mL min^{-1}) produced from 50 mM KH_2PO_4, pH 6 (A), and 50 mM KH_2PO_4, pH 6, containing 30% CH_3CN (B) (t = 0 min, 5% B; t = 20 min, 50% B) had to be used (gradient VII). Routinely, a column 25 cm long with an internal diameter of 0.4 cm was used, the only exception being in the purification of dimethoxytrityl oligonucleotides when these dimensions were 30 × 0.8 cm.

^{31}P NMR spectra were recorded on a Bruker WP200 SY spectrometer operating at 81.01 MHz with quadrature detection and ^1H broad-band decoupling. Samples were contained in 5-mm precision tubes containing a concentric capillary filled with the appropriate reference. Chemical shifts are given in parts per million and are positive when downfield from the standard. Samples soluble in organic solvents were recorded in $CDCl_3$ containing 2% pyridine, and aqueous samples (with the exception of the octanucleotides) were measured in 100 mM EDTA adjusted to pH 8 with NaOH and containing 50% D_2O. These samples were referenced to 85% H_3PO_4. The spectra of the octanucleotides were recorded in 25 mM Hepes–NaOH, pH 7.5, containing 25 mM EDTA, 50 mM NaCl, and 30% D_2O as solvent and trimethyl phosphate as standard. Samples of 1–2 μmol in a total volume of 400 μL were used.

Mass spectra were recorded on a Kratos MS 50S mass spectrometer with a Kratos FAB source in the negative ion mode. The atom gun used xenon and produced a beam of neutral atoms at 8–9 kV. An aqueous solution of the triethylammonium salt of the nucleotide (1–2 μL, containing approximately 20 nmol) was injected into the glycerol matrix (approximately 2 μL) present on the FAB copper probe tip. Water was removed in the direct insertion lock, and the spectra were recorded at a magnet scan rate of 300 s/decade.

Melting curves were recorded in 1-cm cuvettes in a Zeiss DMR 10 spectrophotometer to which a Colora, WK 5 thermostat was attached. The temperature was measured with a Knauer precision temperature bridge and a thermistor enclosed in a glass capillary that extended through the stopper of the cuvette into the solution. Differential melting curves were computed by taking small temperature intervals from the integral recording. All samples were examined at an optical density of between 0.2 and 0.3 at 260 nm in 10 mM Tris-HCl, pH 7.2, 50 mM NaCl, and 10 mM

(p-Chlorophenoxy)acetic Anhydride. (*p*-Chlorophenoxy)acetic acid (46.5 g, 0.25 mol) was dissolved in ethyl acetate (400 mL) and ether (150 mL), and dicyclohexylcarbodiimide (25 g, 0.12 mol) was added. The reaction mixture was stirred at room temperature for 2 h, the precipitated dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness. The crude product was recrystallized from ether (500 mL): yield 60%; mp 90–91 °C [lit. 90–91 °C (van Boom et al., 1971)].

N^6-Benzoyl-3'-O-[(p-chlorophenoxy)acetyl]-2'-deoxyadenosine. N^6-Benzoyl-5'-*O*-(dimethoxytrityl)-2'-deoxyadenosine (5 mmol, 3.62 g) was dissolved in 20 mL of pyridine, and (*p*-chlorophenoxy)acetic anhydride (6 mmol, 2.13 g) was added. The reaction was monitored by TLC [$CHCl_3$–CH_3OH (9:1 v/v); R_f of starting material 0.8, R_f of product 0.95] and was usually complete in 3 h. Occasionally a second addition (2 mmol) of the anhydride was needed to ensure complete reaction. When the reaction was finished, methanol (10 mL) was added and after a further 2 h the solvent was evaporated at a water pump. Pyridine was removed by successive coevaporations with toluene using an oil pump. The dimethoxytrityl group was removed by the addition of 100 mL of an ice-cold solution of 2% *p*-toluenesulfonic acid in $CHCl_3$–CH_3OH (7:3 v/v). After 5 min on ice the mixture was poured into 250 mL of 5% aqueous $NaHCO_3$. The chloroform layer was washed with a further 250 mL of 5% aqueous $NaHCO_3$, 250 mL of saturated NaCl, and 250 mL of water. The N^6-benzoyl-3'-*O*-[(*p*-chlorophenoxy)acetyl]-2'-deoxyadenosine remains in the chloroform layer during these extractions as a fine dispersion. Purification can be simply achieved by filtration and washing of the residue sequentially with water, chloroform, and ether. The product appeared pure by TLC [$CHCl_3$–CH_3OH (8:2 v/v)]: R_f 0.7; yield 90%.

R_P and S_P Diastereomers of 5'-O-(N^6-Benzoyl-2'-deoxyadenosyl) 3'-O-[N^2-Isobutyryl-5'-O-(dimethoxytrityl)-2'-deoxyguanosyl] O-Methyl Phosphorothioate. N^6-Benzoyl-3'-*O*-[(*p*-chlorophenoxy)acetyl]-2'-deoxyadenosine (1 mmol, 493 mg) was dissolved in 1 mL of Me_2SO and 5 mL of THF. N^2-Isobutyryl-5'-*O*-(dimethoxytrityl)-2'-deoxyguanosine 3'-*O*-(morpholinomethoxyphosphite) (1 mmol, 794 mg) was dissolved in 5 mL of THF and added to the solution of the deoxyadenosine compound. Tetrazole (4 mmol, 280 mg) was then added and the mixture set aside for 1 h; then it was poured into a suspension of elemental sulfur (320 mg, 10 mmol) in 10 mL of pyridine and stirred for a further 1 h. The sulfur was then removed by filtration through glass wool and the solution evaporated to an oil with a water pump. Excess pyridine was removed by coevaporation with toluene using an oil pump. The viscous liquid so obtained was dissolved in 50 mL of $CHCl_3$ and the solution extracted twice with 50 mL of 5% aqueous $NaHCO_3$ and twice with 50 mL of saturated NaCl. The $CHCl_3$ layer was dried over Na_2SO_4 and evaporated to dryness. At this stage TLC [$CHCl_3$–CH_3OH (9:1 v/v)] showed the product (R_f 0.8) as the main component together with unreacted deoxyadenosine starting material (R_f 0.7) and some trityl positive material at the origin as minor contaminants. The 3'-*O*-[(*p*-chlorophenoxy)acetyl] protecting group was removed by dissolving the crude product in dioxane (16 mL) and 25% aqueous NH_3 solution (4 mL) and setting it aside at room temperature for 90 min. The R_f value of the desired product in the above TLC system is 0.5. The final product was purified by passage over a column of silica gel 60 equilibrated with $CHCl_3$–CH_3OH–pyridine (95:4:1 v/v) and eluted with the same solvent under a positive nitrogen pressure of 0.7 atm. Fractions of 10 mL were collected and analyzed by TLC on silica gel plates with concentrating zones, eluted with $CHCl_3$–CH_3OH–pyridine (92:7:1 v/v). Fractions 76–85 (fast diastereomer) and 87–97 (slow diastereomer) were pooled and evaporated to dryness. As detailed below, the fast and slow isomers have the *S* and *R* configurations at phosphorus, respectively. Both diastereomers appeared ≥95% in the above TLC system with R_f values of 0.4 and 0.3 for the fast and slow isomers, respectively. Additionally, both isomers were ≥95% pure by ^{31}P NMR spectroscopy. δ values of 69.67 and 69.86 were found for the fast and slow isomers, respectively, when measured in $CDCl_3$–pyridine (98:2 v/v). Each diastereomer was obtained in yields of between 25 and 35% (overall yield between 50 and 70%). In order to establish the absolute configurations of the two diastereomers, a mixture of the fast and slow isomers (1:3 equiv of each) was completely deblocked as follows. Approximately 25 mg of the mixture was dissolved in dioxane (200 μL), triethylamine (100 μL), and thiophenol (100 μL) and left at room temperature for 90 min. The dimer was then precipitated with petroleum ether and the precipitate triturated with 3 × 10 mL of petroleum ether to remove excess thiophenol. Solvent was removed by evaporation, the precipitate dissolved in 2 mL of 25% aqueous ammonia, and the solution heated at 50 °C for 5 h. The ammonia was removed under reduced pressure and the residue taken up in 500 μL of 80% acetic acid and set aside at room temperature for 1 h. The acetic acid was removed by several coevaporations with water, the product obtained was dissolved in 2 mL of water, and the solution was extracted 3 times with 5 mL of ether. The aqueous layer was evaporated to dryness and the residue dissolved in a small volume of water. Reverse-phase HPLC (gradient VI) of the product revealed two peaks in a ratio of 3:1 eluting at 2.5 and 4 min, respectively, which coeluted with a standard mixture of the two diastereomers of d[Gp(S)A]. The later-eluting small peak in the HPLC was completely digested by nuclease P1, giving dG and dAMPS, whereas the early large peak was not hydrolyzed by this enzyme. ^{31}P NMR spectroscopy of the completely deblocked dinucleoside phosphorothioate showed two resonances of 3:1 intensity at δ 55.88 and 54.86, respectively.

5'-O-[N^6-Benzoyl-3'-O-(morpholinomethoxyphosphino)-2'-deoxyadenosyl] 3'-O-[N^2-Isobutyryl-5'-O-(dimethoxytrityl)-2'-deoxyguanosyl] O-Methyl Phosphorothioate. $DMTdG^{ib}p(S,OCH_3)dA^{bz}_{OH}$ (350 μmol, 350 mg, diastereomerically pure) was dissolved in 5 mL of CH_2Cl_2 (freshly passed over basic alumina to remove acidic contaminants) in a 10-mL flask, and diisopropylethylamine (1.4 mmol, 250 μL) was added. The flask was sealed with a rubber septum, flushed with dry nitrogen, and cooled to 0 °C. Morpholinomethoxychlorophosphine (700 μmol, 100 μL) was added with a syringe and the mixture left on ice for 30 min. Ethyl acetate (10 mL, prewashed with 5% $NaHCO_3$) was added and the mixture extracted with 10 mL of 5% $NaHCO_3$ followed by 10 mL of saturated NaCl. The organic phase was applied directly to a column (10 × 2.5 cm) of silica gel 60 (230–400 mesh) equilibrated with EtOAc–CH_3CN–NEt_3 (7:2:1 v/v), and products were eluted with this solvent under a positive nitrogen pressure of 1 atm. Fractions of 5 mL were collected, and those containing product (fractions 6–25) were pooled and evaporated to dryness. Yields of 70% were typically obtained, and the product appeared to be about 90% pure by TLC [$CHCl_3$–CH_3OH–NEt_3 (9:0.5:0.5 v/v) or EtOAc–CH_3CN–NEt_3 (7:2:1); R_f 0.75 in each case] and was used without further characterization.

Solid-Phase Oligonucleotide Synthesis. Oligonucleotides were synthesized in a 2-mL glass syringe fitted with a glass

frit (porosity of 3) and a cm long needle (Tanaka & Letsinger, 1982). The syringe was charged with 90 mg of silica gel containing 10 μmol of bound N^4-anisoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidine. The 5′-*O*-(dimethoxytrityl) 3′-(morpholinomethoxyphosphite) derivatives of N^4-anisoyl-2′-deoxycytidine, N^6-benzoyl-2′-deoxyadenosine, and N^2-isobutyryl-2′-deoxyguanosine were used to prepare the nucleotide chain. In all cases, additions to and expulsions from the syringe were made via the needle.

The following synthesis cycle was used: (1) Wash with 1,2-dichloroethane (2 × 2 mL); (2) detritylate by addition of 2 mL of 10% solution of trichloroacetic acid in 1,2-dichloroethane for 2 min; (3) wash with 1,2-dichloroethane (3 × 2 mL); (4) render anhydrous by washing with acetonitrile (10 × 2 mL); (5) couple by addition of 100 μmol of the appropriate 5′-*O*-(dimethoxytrityl) nucleoside 3′-*O*-(morpholinomethoxyphosphite) in 0.5 mL of acetonitrile together with 250 μmol of tetrazole in 0.5 mL of acetonitrile (coupling times were 30 min for the first cycle and 10 min for subsequent cycles); (6) wash with acetonitrile (2 × 2 mL); (7) oxidize by addition of 1 mL of a 1% solution of iodine dissolved in lutidine–THF–H_2O (1:8:1 v/v) for 1 min; (8) wash with acetonitrile (3 × 2 mL); (9) cap unreacted hydroxyl groups by addition of 1 mL of a 10% solution of (dimethylamino)pyridine in THF, 0.25 mL of lutidine, and 0.25 mL of acetic anhydride for 5 min; (10) wash with acetonitrile (3 × 2 mL). Step 10 completes the addition of one nucleotide. The growing oligomer is further elongated by beginning again at step 1.

Phosphorothioate-containing oligomers were prepared by two methods. The first method was the addition of elemental sulfur to the phosphite intermediate, resulting ultimately in a mixture of diastereomers of the phosphorothioate oligomer prepared. In this case, after nucleotide coupling (step 5) the silica gel was washed with THF (3 × 2 mL) and a suspension of elemental sulfur (100 mg) in pyridine (2 mL) added. This addition was made with a Pasteur pipet after removing the syringe piston. The piston was then replaced and the syringe and contents were gently shaken for 2 h. Excess pyridine was expelled and the sulfur removed by the uptake and expulsion of 2 mL of a 50:50 mixture of CS_2–pyridine. Elemental sulfur is soluble in this mixture and four cycles are enough to ensure its removal. The silica gel was then washed with pyridine (4 × 2 mL) and the synthesis cycle continued at step 8. The second method used to produce phosphorothioate oligomers was the addition of a chirally pure $DMTdG^{ib}p(S,OCH_3)dA^{bz}_{mmp}$ dimer instead of a monomer. In this case, the only alteration in the protocol was an increase in the coupling time to 45 min. After the addition of the last nucleotide the synthesis cycle was terminated with the completion of step 8. The methyl groups were removed from the phosphotriester in the syringe by the addition of 2 mL of dioxane–NEt_3–thiophenol (2:1:1 v/v) for 1 h. This solution was then expelled and the silica gel washed with methanol (3 × 2 mL) and then ether (3 × 2 mL). The syringe piston was removed and the silica gel dried by the careful passage of nitrogen (entry via the barrel, exit via the needle) through the gel bed. Thus dried, the silica gel was easily poured into a 25-mL round-bottomed flask. The ligomer was cleaved from the silica gel, and the base-protecting groups were simultaneously removed by adding 3 mL of 25% aqueous ammonia and heating at 50 °C for 15 h. After this tim the ammonia solution was removed by evaporati n at a water pump. Care should be taken with this step as this solution has a tendency to froth. The product was dissolved in 1 mL of a 1% aqueous NEt_3 solution and silica gel removed by filtration through a small plug of glass wool in a Pasteur pipet. The filtrate was extracted with ether (5 × 2 mL), briefly evaporated at a water pump to remove excess ether, and made up to about 1 mL with aqueous 1% NEt_3. The dimethoxytrityl oligomer so produced was purified by reverse-phase HPLC using gradient I (retention time, 8.7 min). Usually ten aliquots of 100 μL each were injected onto the column. The fractions that contained product were pooled and evaporated to an oil at a water pump. Most of the TEAA was removed with a high-vacuum pump and repeated coevaporations with methanol. During these evaporations some detritylation occurred. The dimethoxytrityl groups were then completely removed by a 1-h treatment with 2 mL of 80% acetic acid. The acetic acid was removed by evaporation, the resulting oligomer was dissolved in 1 mL of water, and the solution was extracted with ether (5 × 2 mL). Excess ether was removed by a brief evaporation of the aqueous phase and the product made up to a volume of 1 mL. Final purification, by injection of ten aliquots of 100 μL each, was by reverse-phase HPLC using gradient II. Fractions that contained product were pooled and evaporated to dryness. Excess TEAB was removed by coevaporation from methanol. The purity of the oligonucleotides was checked by HPLC using gradients III, IV, and V. The purified products were dissolved in 1 mL of water and stored frozen at −20 °C. Usually between 1.5 and 3 μmol of pure octanucleotides was obtained. This represents a yield of between 15 and 30% based on the first cytidine residue attached to the silica gel.

5′-Phosphorylation of Oligonucleotides. The appropriate oligonucleotide (about 2 A_{260} units) dissolved in a 200-μL volume containing 50 mM glycine, pH 9.2, 10 mM DTT, 5 mM $MgCl_2$, and 1 mM ATP was phosphorylated with polynucleotide kinase (25 units) at 37 °C. The reacti n was monitored by HPLC (gradient II) and was usually complete in 90 min. The 5′-phosphorylated oligomers were then isolated by preparative HPLC (gradient II).

Digestion of Oligonucleotides with Nuclease P1 and Alkaline Phosphatase. The appropriate oligomer (about 1 A_{260} unit) was dissolved in 200 μL of 25 mM Tris-HCl, pH 7, and digested with nuclease P1 (20 μg) for 2 h at 37 °C. Digestion was complete after this time and an aliquot was analyzed by HPLC (gradient VI). To the remaining solution were added $MgCl_2$ (to a final concentration of 10 mM) and alkaline phosphatase (10 μg). After a further 2-h incubation at room temperature the mixture was again analyzed by HPLC (gradient VII).

Desulfurization of Phosphorothioate-Containing Oligonucleotides. About 0.5 A_{260} unit of the phosphorothioate-containing oligomer dissolved in H_2O (25 μL) was reacted with iodine (0.5 mg) dissolved in pyridine (75 μL). The reaction was carried out at room temperature for 45 min. Water (1 mL) was then added and the iodine extracted with ether (5 × 2 mL). The aqueous phase was then evaporated to dryness, the residue redissolved in 100 μL of H_2O, and the product of the reaction purified by HPLC (gradient II).

Digestion of Oligonucleotides with EcoRI. Oligonucleotide (approximately 1 A_{260} unit) dissolved in a 200-μL volume containing 10 mM Tris-HCl, pH 7.6, 80 mM NaCl, and 20 mM $MgCl_2$ was digested with *Eco*RI (between 2.75 and 13.75 μg). The reaction mixtures were incubated at 16 °C for times of up to 24 h. Aliquots were analyzed by HPLC, and in the cases where cleavage took place the products were purified by HPLC (gradient II).

Results

The octamers were synthesized by the phosphite method on a solid support empl ying deoxynucleoside 3′-*O*-(methoxy-

morpholinophosphites) (Matteucci & Caruthers, 1981; McBride & Caruthers, 1983; Dörper & Winnacker, 1983) as the building units and Fractosil 200 (a silica-based material) as the solid phase, contained in a glass syringe (Tanaka & Letsinger, 1982). Some of the difficulties encountered in the synthesis of the all-phosphate-containing octamer d-(GGAATTCC) by this method with respect to coupling times and removal of dimethoxytrityl groups will be discussed under Discussion.

To obtain a mixture of the R_P and S_P diastereomers of the phosphorothioate-containing octamer d(GGsAATTCC), the iodine–water oxidation step of the phosphite group between dA and dG was replaced by one consisting of the addition of elemental sulfur. Routinely, a suspension of sulfur in pyridine and a 2-h reaction time were used. After reaction the insoluble sulfur was removed by flushing with a CS_2–pyridine (1:1) solution. Addition of sulfur in a homogeneous solution in this solvent was tried as an alternative and gave comparable results. The addition of sulfur instead of the oxidation did not reduce the yield of the subsequent coupling step, which was ≥95% as determined spectroscopically by the liberation of the dimethoxytrityl group.

In order to prepare chirally pure oligomers, we have utilized the addition of a presynthesized chirally pure phosphorothioate dimer to the growing oligonucleotide chain. The phosphorothioate-containing d[Gp(S)A] dimer was prepared by condensing N^2-isobutyryl-5′-*O*-(dimethoxytrityl)-2′-deoxyguanosine 3′-(methoxymorpholinophosphite) with N^6-benzoyl-3′-*O*-[(*p*-chlorophenoxy)acetyl]-2′-deoxyadenosine using tetrazole as the activating agent. Subsequent addition of elemental sulfur yielded the fully protected phosphorothioate dimer, and a brief treatment with ammonia then removed the (*p*-chlorophenoxy)acetyl-protecting group. Purification and diastereomer separation were simultaneously achieved by silica gel chromatography. It is important to use silica gel 60 H for this separation and also the solvent mixture given under Materials and Methods. Other silica gel types and solvent systems were much less effective in diastereomer resolution. The fast and slow fractions of the required dimer product appeared pure by TLC after silica gel chromatography. Additionally, both fractions appeared pure by ^{31}P NMR spectroscopy (fast, δ 69.67; slow, δ 69.86). ^{31}P NMR spectroscopy of a 3:1 slow:fast mixture confirmed that this difference in chemical shifts was real and that the fast isomer resonates at higher field. The absolute configuration at phosphorus of the two fractions was established by the complete deblocking of a small sample of a 3:1 slow:fast mixture. Removal of the methyl groups with thiophenol occurs with C–O bond cleavage and so does not change the configuration at phosphorus (Daub & van Tamelen, 1977). ^{31}P NMR spectroscopy of the resulting mixture after this deblocking revealed two peaks in a 3:1 ratio at δ 55.89 and 54.87, respectively. Since it is known that the S_P diastereomer of dinucleoside phosphorothioates resonates at higher field than the R_P diastereomer (Romaniuk & Eckstein, 1982; Bartlett & Eckstein, 1982), this establishes that the fast fraction contained the isomer with the S_P configuration and the slow fraction the ne with the R_P configurati n. Confirmation of this result comes from reverse-phase HPLC of th deblocked mixture in which the maj r peak elutes before the minor. Again the R_P diastereomer f dinucleoside phosphorothioates is known to elute before the S_P in reverse-phase HPLC systems (Romaniuk & Eckstein, 1982; Bartlett & Eckstein, 1982). Finally, the major product was susceptible to digestion by snake venom phosphodiesterase but not by nuclease P1, whereas

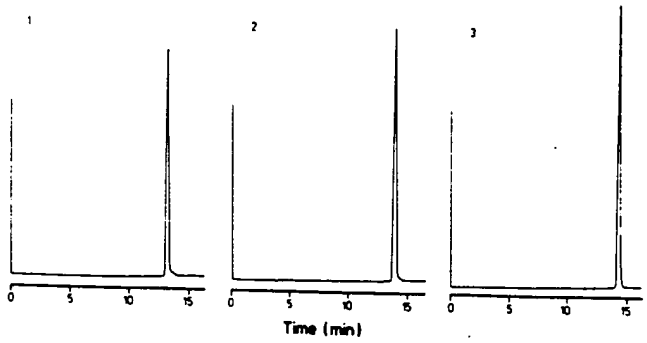


FIGURE 1: Reverse-phase HPLC analysis of octamers: (1) d-(GGAATTCC), (2) (R_P)-d(GGsAATTCC), and (3) (S_P)-d-(GGsAATTCC); gradient III was used.

the opposite enzyme selectivity was seen for the minor product. Again this means that the major peak has the R_P configuration and the minor the S_P (Burgers & Eckstein, 1978b; Potter et al., 1983). All these tests indicate that the slow triester has the R_P and the fast the S_P configuration. After purification the R_P and S_P diastereomers of the methyl esters of d[Gp(S)A] were separately treated with methoxymorpholinochlorophosphite, resulting in a dinucleoside phosphorothioate containing a methoxymorpholinophosphite moiety at the 3′-hydroxyl group. After further purification by silica gel chromatography these methoxymorpholinophosphite dimers can then be attached to the growing nucleotide chain in the usual fashion. Subsequent to the coupling of this dinucleoside phosphorothioate, one more coupling with 2′-deoxyguanosine 3′-*O*-(methoxymorpholinophosphite) and an oxidation step have to be performed to complete the synthesis of the octamer.

After completion of the solid-phase synthesis the methyl-protecting groups were removed from the phosphate and phosphorothioate triesters with thiophenolate. The base-protecting groups were then removed, and the oligonucleotide was simultaneously cleaved from the silica gel by ammonia treatment. The oligonucleotide, containing a dimethoxytrityl group at the 5′-terminus, was then purified by reverse-phase HPLC. All the truncated sequences resulting from incomplete coupling yields followed by capping with acetic anhydride do not contain a highly hydrophobic dimethoxytrityl group and therefore elute much earlier than the desired product on reverse-phase HPLC. The purified dimethoxytrityl oligomer was then treated with acetic acid to remove the dimethoxytrityl-protecting group and finally purified further by HPLC. For this final purification TEAB was used as the buffer salt in conjunction with an acetonitrile gradient. All the components used in this step are volatile and are easily removed by evaporation, eliminating the need for a final desalting step.

The purified oligonucleotides were 5′-phosphorylated by using polynucleotide kinase with ATP as the phosphoryl donor. This reaction was monitored by reverse-phase HPLC, as the phosphorylated products elute before the starting octamers. The same system was used for the purification of the 5′-phosphorylated oligonucleotides.

The purity of the oligomers produced has been checked by reverse-phase HPLC using either KH_2PO_4, TEAA, or tetrabutylammonium phosphate as buffer with an acetonitrile gradient. In all these systems d(GGAATTCC) as well as (S_P)- and (R_P)-d(GGsAATTCC) appeared ≥95% pure (Figure 1). The all-oxygen-containing compound always eluted earlier than the phosphorothioate-containing oligomers with base line separation being achieved. No separation was observed between the S_P and R_P isomers of d(GGsAATTCC), which when coinjected eluted as a single symmetrical peak. The 5′-

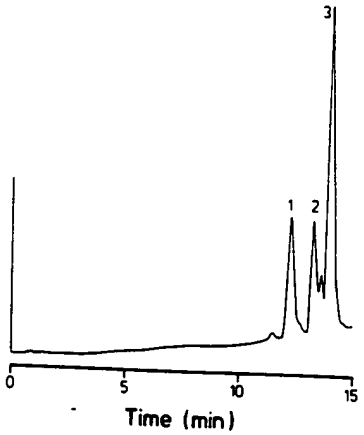


FIGURE 2: Reverse-phase HPLC analysis of 5′-phosphorylated octamers. The solution was prepared by mixing solutions containing the individual isomers: (1) d(pGGAATTCC), (2) (S_P)-d(pGGsAATTCC), and (3) (R_P)-d(pGGsAATTCC); gradient V was used. The small peak between peaks 2 and 3 is due to a contaminant in (R_P)-d(GGsAATTCC).

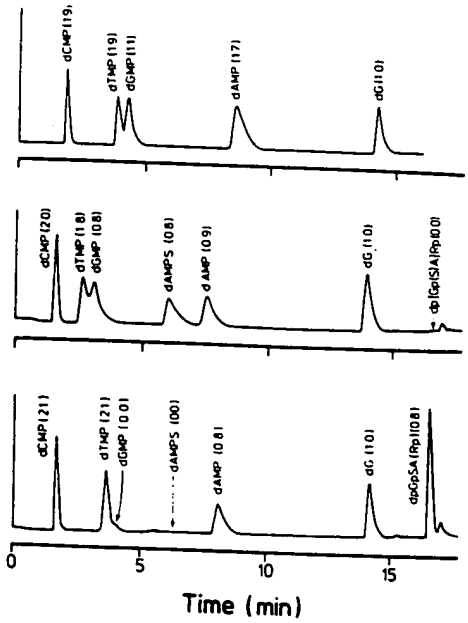


FIGURE 3: HPLC analysis of nuclease P1 digest of octamers: digests of d(GGAATTCC) (top), (R_P)-d(GGsAATTCC) (middle), and (S_P)-d(GGsAATTCC) (bottom); gradient VI was used. The numbers in parentheses refer to the equivalent amounts of each of the nucleotides by integration.

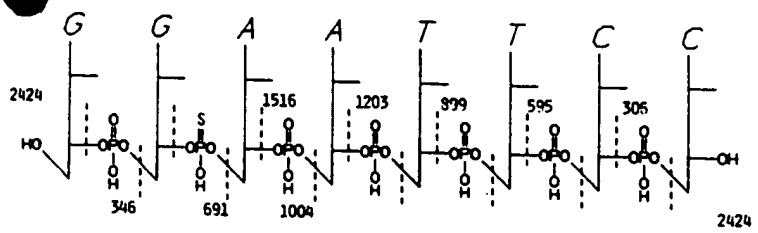
phosphorylated oligomers were also analyzed by reverse-phase HPLC using the KH_2PO_4 and TEAA buffer systems. d(pGGAATTCC) and (S_P)-d(pGGsAATTCC) appeared ≥95% pure. As for the nonphosphorylated octamers, the all-oxygen-containing nucleotide eluted before those containing sulfur. Remarkably, the two phosphorylated phosphorothioate oligonucleotide diastereomers were base line resolved, with the S_P isomer eluting before the R_P (Figure 2). The individual 5′-phosphorylated phosphorothioate diastereomers showed negligible contamination with the other isomer, indicating that the original unphosphorylated oligomers must also have been of a very high diastereomeric purity.

The oligonucleotides were further characterized by nuclease P1 digestion followed by analysis of the products by HPLC. Nuclease P1 cleaves nucleotides giving nucleoside 5′-phosphates and so d(GGAATTCC) would be expected to yield dG, dGMP, dAMP, dTMP, and dCMP in a ratio of 0.5, 0.5, 1.0, 1.0, and 1.0, respectively. This is indeed the case as is shown (Figure 3). Further treatment of this mixture with alkaline phosphatase gave dG, dA, dT, and dC in the expected equimolar ratios. Phosphorothioates having the S_P configuration are digested by nuclease P1 (Potter et al., 1983; S. Spitzer and F. Eckstein, unpublished results), and so, for example, (S_P)-d[Gp(S)A] would yield dG and dAMPS. Phosphorothioates having the R_P configuration are not cleaved by nuclease P1 (Potter et al., 1983). Thus (S_P)-d(GGsAATTCC) would be expected to give dG (0.5), dGMP (0.5), dAMPS (0.5), dAMP (0.5), dTMP (1.0), and dCMP (1.0) after nuclease P1 treatment (the figures in parentheses refer to the equivalents expected) as is indeed found (Figure 3). After the addition of alkaline phosphatase the 5′-monophosphates were converted to deoxynucleosides, giving the ratios expected. dAMPS is inert to alkaline phosphatase and so remains unchanged. Treatment of (R_P)-d(GGsAATTCC) with nuclease P1 gave dG (0.5), (R_P)-d[pGp(S)A] (0.5), dAMP (0.5), dTMP (1.0), and dCMP (1.0) as expected and shown (Figure 3). The identification of d[pGp(S)A] in Figure 3 is tentative as we d not possess this compound as a standard. After alkaline phosphatase treatment, however, the (R_P)-d[Gp(S)A] formed can be conclusively identified by comparison with standard material. Additionally, the other dephosphorylated nucleosides were produced in the expected ratios. This analysis not only establishes that the base composition of the synthesized octamers is correct but also proves that the phosphorothioate oligomer of the S_P configuration is derived from the S_P-protected d[Gp(S)A] dimer and that the R_P oligomer is derived from the R_P dimer. This analysis therefore confirms that very little, if any, epimerization at phosphorus occurs during the entire synthesis, deblocking, and purification of the phosphorothioate oligomers.

Treatment of the phosphorothioate-containing oligomers with iodine in pyridine resulted in desulfurization and formation of the normal all-phosphate-containing nucleotide. This reaction was monitored by HPLC and appeared to be both quantitative and free from side reactions. All the d(GGsAATTCC) was converted to a product identical with d(GGAATTCC) by use of several HPLC systems. Further proof of the integrity of the d(GGAATTCC) so produced was that it was completely digested by *Eco*RI, yielding the expected products d(GG) and d(pAATTCC). A similar desulfurization of a [pAp(S)U] copolymer with iodine has previously been reported (Burgers & Eckstein, 1979).

The midpoint of thermal transition of d(GGAATTCC) and the two phosphorothioate oligomers all lie between 23 and 25 °C. Thus, within the experimental error of the method, the various oligomers have similar, if not identical, thermal stabilities.

The negative ion FAB mass spectrum of the "fast" isomer of d(GGsAATTCC) together with the expected breakdown shown in the structural formula is given in Figure 4. The data are summarized in Table I. The background of the spectrum is higher than those given in Grotjahn et al. (1982) for all-phosphate-containing oligomers. The main reason for this background seems to be the smaller homogeneity as well as the incomplete exchange of Na^+ ions for triethylammonium ions, as can be seen from the molecular and sequence ions that are accompanied throughout by the corresponding Na^+-containing masses. The deprotonated molecular ion appears at 2423 d. The 5′-phosphate sequence ions could be registered up t the fifth nucleotide and and 3′-phosphate sequences up to the third. More sequence ions could not be assigned since they are buried in the background. The sequence ions are usually accompanied by relatively intense −18 d (loss of H_2O) and Na^+-containing 22 d ($-H, +Na^+$) ions.



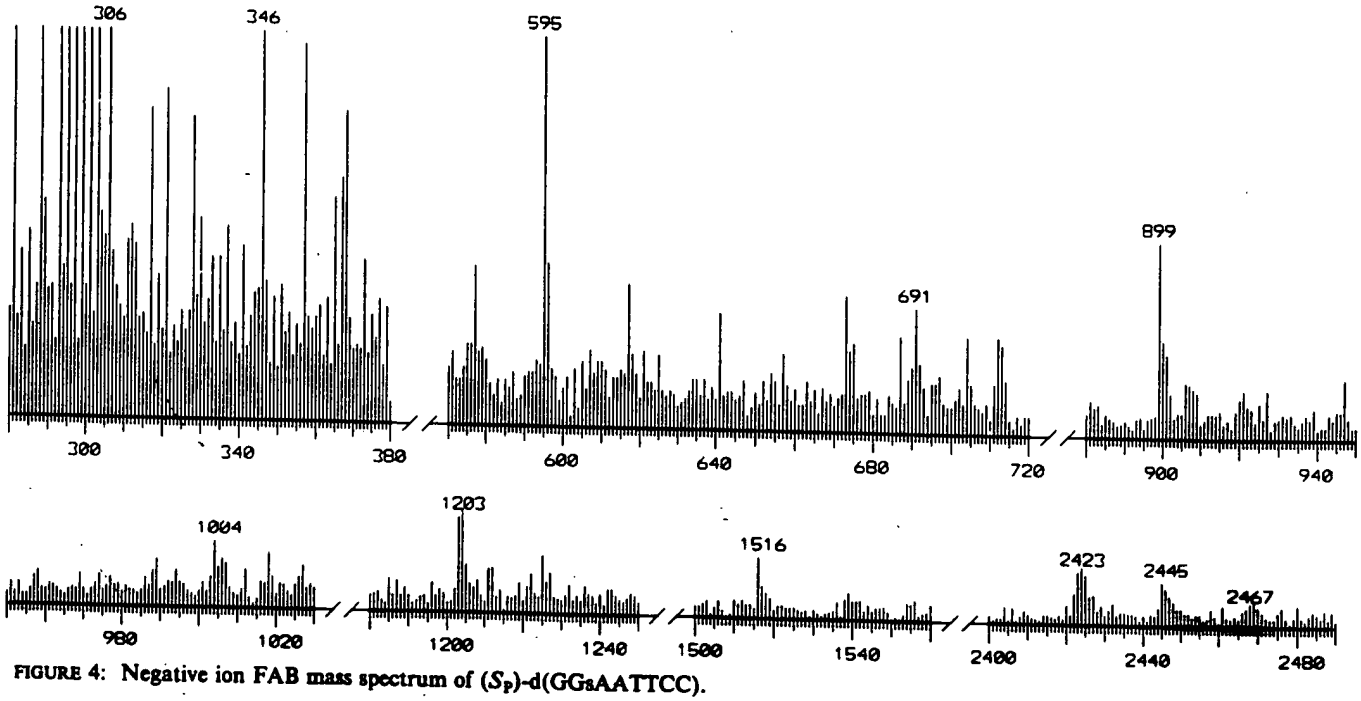


FIGURE 4: Negative ion FAB mass spectrum of (S_P)-d(GGsAATTCC).

Table I: Masses of Sequence Ions Observed in FAB Mass Spectrometric Analysis of d(GGAATTCC) and d(GGsAATTCC)

	d(GGAATTCC)[a]		d(GGsAATTCC)[b]	
	5′-P	3′-P	5′-P	3′-P
(1) sequence ion	306	346	306, 288 ($-H_2O$), 328 (−H + Na)	346, 328 ($-H_2O$), 368 (−H + Na)
(2) sequence ion	595	675	595, 577 ($-H_2O$), 617 (−H + Na)	691, 673 ($-H_2O$), 713 (−H + Na)
(3) sequence ion	899	988	899, 881 ($-H_2O$), 921 (−H + Na)	1004, 986 ($-H_2O$), 1026 (−H + Na)
(4) sequence ion	1203	1301	1203, 1185 ($-H_2O$), 1225 (−H + Na)	
(5) sequence ion	1516	1605	1516, 1538 (−H + Na)	
(6) sequence ion	1829	1909		
(7) sequence ion	2158	2198		
(8) deprotonated molecular ion	2407		2423, 2445 (−H + Na), 2467 (−2H + 2Na)	

[a] Data taken from a spectrum not shown. [b] Data from the spectrum shown in Figure 4.

The ^{31}P NMR spectrum of d(GGAATTCC) taken at 10 °C shows a group of resonances between δ −3.9 and −4.5 (Figure 5). The spectrum of (S_P)-d(GGsAATTCC) shows in addition a signal of intensity 1.0 at δ 50.76 representing the resonance of the phosphorothioate group. Only one signal is observed, indicating high diastereomeric purity. The spectrum of the mixture of diastereomers of d(GGsAATTCC) shows two resonances at δ 51.16 and 50.74 in an approximate ratio of 1:1, confirming what was found from the nuclease P1 digest f the mixture of diastereomers, namely, that sulfur addition to the oligonucleotide proceeds without any detectable stereoselectivity.

Both d(GGAATTCC) and d(pGGAATTCC) were digested by *Eco*RI to give only two products as monitored by HPLC. Collection of these products and analysis using the nuclease P1 and nuclease P1–alkaline phosphatase treatment showed that these products were d(GG) and d(pAATTCC) in the case of d(GGAATTCC) and d(pGG) and d(pAATTCC) in the case of the phosphorylated derivative. The 5′-phosphorylated octamer was hydrolyzed faster than d(GGAATTCC). Thus, under our standard conditions as described under Materials and Methods using approximately 13.75 μg of enzyme, the cleavage of the phosphorylated octamer had proceeded to about 50% in 1 h whereas cleavage of the unphosph rylated octamer had only occurred to about 6%. Treatment of the R_P isomers f both d(GGsAATTCC) and d(pGGsAATTCC) with *Eco*RI also resulted in cleavage at a single point with two products being formed (Figure 6). Collection and analysis f the products sh wed that they were d(GG) and d[p(S)-AATTCC] in the case f the unphosphorylated oligomer and d(pGG) and d[p(S)AATTCC] for the phosphorylated species. Again the 5′-phosphorylated octamer was cleaved at a faster rate than the unphosphorylated octamer. With the same amount of enzyme as above the reaction with the former had

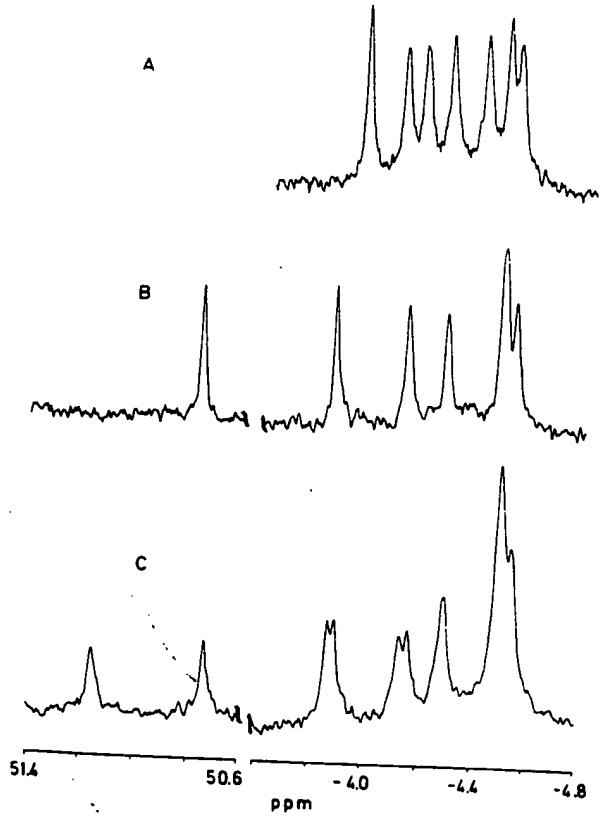


FIGURE 5: ^{31}P NMR spectra of octamers: (A) d(GGAATTCC); (B) (S_P)-d(GGsAATTCC); (C) mixture of (R_P)- and (S_P)-d(GGsAATTCC). Spectra were recorded at 10 °C. Parameters were as follows: offset, 1350 (A) and 3800 Hz (B and C); sweep width, 800 (A) and 6024 Hz (B and C); pulse width, 5.5 (A) and 4.0 μs (B and C); 16K (A) and 32K (B and C); acquisition time, 10.24 (A) and 2.7 s (B and C); line broadening, 0.4 (A) and 0.5 Hz (B and C); number of transients, 916 (A), 1530 (B), and 1000 (C). Chemical shifts are relative to trimethyl phosphate.

reached 75% completion after 20-h incubation, whereas reaction with the latter had only occurred to 10% completion. Because of the scarcity of material no detailed kinetic studies could be undertaken with any of the octamers so that all the values given here obtained by measurement of one or two time points are only approximate.

The S_P isomers of d(GGsAATTCC) and d(pGGsAATTCC) were not cleaved even after incubation for 30 h.

Discussion

The observation that at least certain restriction endonucleases including *Eco*RI can cleave phosphorothioate internucleotidic linkages albeit more slowly than phosphate linkages (Vosberg & Eckstein, 1982; B. V. L. Potter, H. P. Vosberg, and F. Eckstein, unpublished results) led us to attempt the synthesis of an oligonucleotide containing the recognition sequence for such an enzyme and possessing a phosphorothioate group at the site of cleavage. It was envisaged that such a compound would be a substrate and should be suitable for the determination of the stereochemical course f the enzyme reaction, empl ying methods that have been applied to a large range of phosph ryl and nucleotidyl transferring enzymes [see review for Eckstein (1983a,b)]. The most suitable enzyme for study seemed to be the enzyme *Eco*RI since in the class of restriction endonucleases it is the most thoroughly investigated, and thus any information on the stereochemistry of the reaction would be a most useful additional detail for the description of a mechanism (Modrich,

FIGURE 6: HPLC analysis of a partial *Eco*RI digestion of (R_P)-d(pGGsAATTCC): (1) d(GG), (2) d[p(S)AATTCC], and (3) (R_P)-d(GGsAATTCC); gradient II was used; incubation conditions as described under Materials and Methods.

1982). Moreover, it is an enzyme most easily obtainable in sufficient quantities due to the existence of an overproducing strain. This enzyme is known to cleave the octanucleotides d(pTGAATTCC) (Greene et al., 1975) and d(pGGAATTCC) (Goppelt et al., 1980). The latter has a higher thermal stability than the former, and it was thus decided to synthesize the octanucleotide d(GGsAATTCC) that contains the recognition sequence for *Eco*RI with a phosphorothioate between dG and dA, the position of cleavage.

Modern methods of oligonucleotide synthesis are based on either the phosphotriester (Marugg et al., 1983; Gait et al., 1982a,b; Ito et al., 1982; Köster et al., 1983) or the phosphite methodologies (Matteuci & Caruthers, 1981). In the latter a phosphite internucleotidic linkage is formed first, which is oxidized in a second step with iodine–water to a phosphate linkage. It has been shown in solution that such dinucleoside phosphite triesters can be converted to the corresponding dinucleoside phosphorothioate triesters by addition of sulfur instead of oxidation with iodine–water (Burgers & Eckstein, 1978a; Marlier & Benkovic, 1980). Additionally, deblocking of methyl phosphotriesters by thiophenol proceeds with C–O bond cleavage (Daub & van Tamelen, 1977), eliminating the possibility of epimerization at phosphorus during triester to diester conversion. Thus, the phosphite methodology seemed very attractive for the synthesis of the modified octamer as very little modification of the existing methodology was needed.

The phosphite approach based on morpholinometh xyphosphine was chosen as the starting materials are easy t prepare and purify and, in addition, are very stable when dissolved in the usual solvents used for oligonucleotide synthesis (McBride & Caruthers, 1983; Dörper & Winnacker, 1983). Furthermore, solid-phase synthetic methods, as opposed t those conducted in solution, greatly simplify both the synthesis and the subsequent purification of the oligomers prepared (Matteuci & Caruthers, 1981). With these considerations in mind we chose to prepare the desired phosphorothioate-containing octanucleotide and also, as a control, the corresponding all-phosphate-containing octamer by a solid-phase method using nucleoside methoxymorpholinophosphites as building blocks.

During the synthesis of d(GGAATTCC), which has also been synthesized by a polymer-supported phosphotriester ap-

proach earlier (Oktsuka et al., 1982), we noticed that the times required to couple the incoming nucleoside methoxymorpholinophosphites to the free 5'-hydroxyl group, using tetrazole as the activating agent, were somewhat greater than those recommended for the nucleoside methoxydimethylaminophosphite method. For this latter method a 5-min reaction time suffices, whereas all couplings except the initial one using the morpholino derivative required 10 min to go to completion. The first coupling appeared to be especially slow, and 30 min was necessary in order to obtain ≥95% coupling yields. A similarly slow initial coupling step was found when nucleoside methoxydiisopropylaminophosphines and silica gel were used as the solid support (Adams et al., 1983). These authors suggested that steric hindrance was the cause of this slow reactivity and showed that changing the support to controlled pore glass appeared to overcome this problem. Recently, McBride & Caruthers (1983) have demonstrated that nucleoside methoxymorpholinophosphines are less reactive than the corresponding dimethylamino derivatives. Fröhler & Matteucci (1983) showed that the use of (*p*-nitrophenyl)-tetrazole, instead of tetrazole, as the activating agent greatly speeded up the reaction rates with nucleoside methoxymorpholinophosphites. Presumably, incorporation of these two modifications will drastically reduce the total synthesis time. Additionally, we have found that the use of $ZnBr_2$ as the detritylating agent is unsatisfactory. This Lewis acid was suggested as a replacement for protic acids as it does not cause depurination of N^6-benzoyldeoxyadenosine residues under conditions where it removed dimethoxytrityl groups (Matteucci & Caruthers, 1981). However, we have observed detritylation with this reagent (used as a saturated solution in CH_3NO_2–CH_3OH, 95:5) to be slow and incomplete, especially for the sequence DMTdCC. The use of 10% trichloroacetic acid dissolved in dichloroethane for 2 min caused complete detritylation without significant depurination (Gait et al., 1982). Providing that the coupling times mentioned above and under Materials and Methods are followed and trichloroacetic acid is used to remove dimethoxytrityl groups, each coupling step proceeds ≥95% yield, as judged by dimethoxytrityl cation release.

Two alternative approaches were considered for the synthesis of d(GGsAATTCC). First, addition of sulfur instead of oxygen to the growing oligonucleotide chain at the stage when the crucial dG–dA phosphite linkage had been synthesized, and second, addition of a preformed and suitable protected stereochemically pure d[Gp(S)A] derivative as a block. The first approach is attractive because of its simplicity but has the disadvantage that the stereochemistry of addition of sulfur cannot be controlled. Since it originally seemed highly unlikely that the mixture of diastereomers produced could be separated by any chromatographic method, this method was initially used to produce such an octamer quickly and provide material to study various properties, particularly those important for separation and characterization. The second method offered the advantage that if $DMTdG^{ib}p(S,OMe)dA^{bz}_{OH}$ could be separated into its diastereomers and could be added as a block, an octamer containing a phosphorothioate of known configuration would be obtainable, a prerequisite for the envisaged stereochemical studies. Both methods have in common that after introduction of the phosphorothioate triester one additional coupling has to be performed to introduce the terminal dG residue. To ascertain that the iodine–water oxidation step necessary for the formation of this last internucleotidic linkage did not cause desulfurization of the phosphorothioate triester, studies with $DMTdG^{ib}p(OMe,S)dA^{bz}_{OH}$ as a model were undertaken. They showed that even after treatment of up to 1 h with a 1% iodine solution in THF–lutidine–H_2O no such desulfurization occurred. This inertness of the phosphorothioate triesters contrasts with the facile desulfurization of phosphorothioate diesters by iodine dissolved in pyridine.

As expected, the first route produced an octamer comprising a 50:50 mixture of the R_P and S_P isomers of d(GGsAATTCC). This was confirmed by both ^{31}P NMR spectroscopy and digestion with nuclease P1. Supportive evidence for the structure of this phosphorothioate octamer was provided by the desulfurization reaction with iodine. This reaction proceeded in a remarkably clean manner to give the unmodified oligonucleotide d(GGAATTCC), as was evidenced both by HPLC and by its complete cleavage by *Eco*RI to the expected products d(GG) and d(pAATTCC). Although this reaction was not investigated in detail, it became apparent that pyridine is essential for it to proceed. Three other methods are available for the desulfurization of phosphorothioates, namely, the uses of cyanogen bromide (Sammons & Frey, 1982), *N*-bromosuccinimide (Connolly et al., 1982), and bromine (Lowe et al., 1982). With the last two methods side reactions with some of the bases, especially guanosine, occur, which contrasts with the mildness of the iodine method. However, the st reochemical course of the iodine-mediated desulfurization has yet to be determined as it has been for the three other methods.

The second route necessitated the synthesis of $DMTdG^{ib}p(OMe,S)dA^{bz}_{OH}$. One of the problems encountered in this synthesis was the proper choice of intermediary protection of the 3'-OH group of dA. The strategy required that this protecting group had to be removed without hydr lysis of the phosphotriester to allow phosphitylation of the 3'-OH group of dA. The (*p*-chlorophenoxy)acetyl group was selected since it can be removed by brief treatment with dilute ammonia (van Boom et al., 1971), conditions under which the phosphorothioate triester was stable. The two diastereomers of $DMTdG^{ib}p(OMe,S)dA^{bz}_{OH}$ could be separated by silica gel chromatography and their absolute configuration determined after complete deblocking by digestion with nuclease P1, ^{31}P NMR spectroscopy, and HPLC. After reaction with morpholinomethoxychlorophosphine and purification of the products, these two diastereomers could be used as blocks in the octamer syntheses. The final octamers were shown to be diastereomerically pure by ^{31}P NMR spectroscopy and nuclease P1 digestion. Additionally, the nuclease P1 digestion confirmed the expected nucleotide composition. Also, both the R_P and the S_P diastereomers of d(GGsAATTCC) w re desulfurized with iodine to produce d(GGAATTCC), a further proof of the structure.

The mixture of diastereomers produced by the first method and the separate diastereomers produced by the second could both be easily phosphorylated at the 5'-OH groups by polynucleotide kinase. Most remarkable was the finding that although the unphosphorylated diastereomers could not be separated by HPLC, the 5'-phosphorylated species were separable. This is of practical consequence as the first method of synthesis is much easier than the second, allowing the rapid preparation of large amounts of material. Phosphorylation then becomes the handle allowing separation of the mixture of diastereomers produced by this procedure by HPLC.

We felt that a completely independent check ought to be made on the composition and sequence of the phosphorothioate octamer. Normally, a wandering spot sequence analysis should be performed on such an oligonucleotide (Brownlee & Sanger, 1969; Jay et al., 1974). However, the stereoselectivity of most nucleases for one or the other diastereomer f a dinucleoside

phosphorothioate poses problems for the general application of this method to the analysis of phosphorothioate-containing oligomers. It was therefore decided to try FAB mass spectrometry for the analysis of the phosphorothioate octamers as this method has been shown to be capable of analyzing oligonucleotides up to a chain length of ten (Grotjahn et al., 1982). The presence of Na^+, which we were unable to remove by various chromatographic methods, represented one of the main difficulties in this analysis. Nevertheless, the mass spectrum for the S_P isomer (a similar one has been obtained for the R_P isomer) (Figure 4) shows as detailed in Table I that the fragmentation pattern is fully compatible with the structure of the octamer being d(GGsAATTCC). Of particular importance is overlap for the fragmentation from the 3′ and 5′ ends. Fragments from the 3′ end yielding nucleotide 5′-phosphates can be identified up to the fifth nucleotide and those from the 5′ end yielding nucleotide 3′-phosphates up to the third nucleotide including the crucial d[Gp(S)A] part. Thus, this analysis shows that FAB mass spectrometry can successfully be applied to the analysis of modified oligonucleotides. For many modified oligonucleotides this might be the method of choice, particularly for those where the phosphate group has been altered and rendered resistant to nucleases.

A very important characteristic of these octamers is their thermal stability, especially as *Eco*RI requires a double-stranded structure as substrate. This is significant since a decreased thermal stability has been documented for the phosphorothioate analogues of the alternating polynucleotides poly[d(G-C)] and poly[d(A-T)] (Eckstein & Jovin, 1983; Jovin et al., 1983). In these polymers the thermal stability is lowered to the greatest extent when the pyrimidine nucleoside 5′-phosphate is substituted by a phosphorothioate. For poly[d[pGp(S)C]] a decrease in T_m of 8 °C was observed whereas for poly[d[pAp(S)T]] the T_m was lowered by 15 °C. For the polymers containing a purine nucleoside 5′-phosphorothioate such as poly[d[pCp(S)G]] and poly[d[pTp(S)A]] the T_m values were lowered 2 and 5 °C, respectively. However, contrary to this it was found that both (R_P)- and (S_P)-d(GGsAATTCC) as well as the mixture of diastereomers had T_m values of between 23 and 25 °C, similar to that of d(GGAATTCC).

As a further characterization the ^{31}P NMR spectra of all the octamers were recorded. At 10 °C, conditions where these octamers exist as double helices in the buffer system used, the spectrum of d(GGAATTCC) clearly shows seven resonances of the same intensity whereas at 40 °C (not shown), much less resolution was observed. A similar spectrum recorded at 30 °C has been reported by Patel & Canuel (1979). At present we are unable to assign any of these signals to a particular phosphate group in the oligonucleotide sequence. On the basis of what has been observed for the phosphorothioate analogues of poly[d(A-T)] and poly[d(G-C)] (Eckstein & Jovin, 1983; Jovin et al., 1983), it was expected that the spectra of the phosphorothioate and the all-phosphate-containing octamers should be very similar with the exception that the signal arising from the phosphorothioate of d[Gp(S)A] would be missing from this part of the spectrum since the phosphorothioates resonate at much lower field. By this analysis at least one of the phosphate resonances of the unmodified octamer should have been assignable. However, the spectrum of (S_P)-d(GGsAATTCC) recorded at 10 °C does not fit such a pattern. Some resonances seem to be unaltered in the phosphorothioate such as those at δ −4.54, −4.50, −4.34, and −4.15, but more than one have either disappeared or shifted relative to the spectrum of the unmodified octamer. Thus, the resonances of the phosphorothioate octamer at δ −4.54 and −4.50 integrate to 3 rather than 2 equiv. This must be an indication of the changes in conformation caused by the mixture of diastereomers where fine structure is seen in two of the phosphate resonances, indicating differential perturbation presumably of the neighboring phosphates by the two isomers.

As expected, d(pGGAATTCC) was cleaved by *Eco*RI, yielding the appropriate dimer and hexamer. It was also found that the unphosphorylated octamer was a substrate for the enzyme. However, this was cleaved approximately 8 times more slowly than the phosphorylated species. This observation is similar to that of Dwyer-Hallquist et al. (1982), who found in a more detailed study that the enzyme *Hpa*I cleaves d(pGGTTAACC) about 30 times faster than the unphosphorylated octamer. Of the two diastereomers of d(GGsAATTCC) and d(pGGsAATTCC), only the R_P isomers were hydrolyzed. Also, in this case the phosphorylated octamer was cleaved faster by a factor of about 7. These results probably indicate, as suggested by Dwyer-Hallquist et al. (1982), that the 5′-terminal phosphate of this octamer is also part of the recognition sequence of the *Eco*RI enzyme. The observed stereospecificity is in line with what has been found for the hydrolysis by restriction enzymes of enzymatically synthesized DNA-containing phosphorothioate groups in one strand only (Vosberg & Eckstein, 1982; B. V. L. Potter, H. P. Vosberg, and F. Eckstein, unpublished results). In such DNA the phosphorothioate groups are of the R_P configuration, and they were indeed cleaved by the restriction enzymes investigated although at a slower rate than unmodified DNA. Since enzymatic formation of a phosphorothioate internucleotidic linkage by DNA polymerases always produces the R_P configuration (Eckstein, 1983a,b), the stereospecificity of restriction enzymes can only be determined by the chemical synthesis of the phosphorothioate linkage as demonstrated in this publication. The limited kinetic study we were able to carry out indicates that the phosphorylated octamers as well as the unphosphorylated (R_P)-phosphorothioate octamers are cleaved approximately 15 times more slowly than the corresponding all-phosphate-containing octamers. We are at present using (R_P)-d(pGGsAATTCC) to evaluate the stereochemical course of the *Eco*RI catalyzed reaction.

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Registry No. *Eco*RI, 80498-17-5; d(GGAATTCC), 70755-49-6; (R_P)-d(GGsAATTCC), 90584-24-0; (S_P)-d(GGsAATTCC), 90639-14-8; d(pGGAATTCC), 71065-77-5; (R_P)-d(pGGAATTCC), 90584-25-1; (S_P)-d(pGGAATTCC), 90639-15-9; (R_P)-DMTdG^{ib}p(S,OCH$_3$)dA$^{bz}_{mmp}$, 90584-28-4; (S_P)-DMTdG^{ib}p(S,OCH$_3$)dA$^{bz}_{mmp}$, 90639-17-1; (R_P)-DMTdG^{ib}p(S,OCH$_3$)dA$^{bz}_{OH}$, 90584-27-3; (S_P)-DMTdG^{ib}p(S,OCH$_3$)dA$^{bz}_{OH}$, 90639-16-0; (*p*-chlorophenoxy)acetic anhydride, 34359-78-9; (*p*-chlorophenoxy)acetic acid, 122-88-3; N^6-benzoyl-3′-*O*-[(*p*-chlorophenoxy)acetyl]-2′-deoxyadenosine, 90584-26-2; N^6-benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxyadenosine, 64325-78-6; N^2-isobutyryl-5′-*O*-(dimethoxytrityl)-2′-deoxyguanosine 3′-*O*-(morpholinomethoxyphosphine), 86030-51-5; morpholinomethoxychlorophosphine, 86030-42-4; N^4-anisoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidine 3′-*O*-(morpholinomethoxyphosphine), 90584-29-5.

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APPENDIX D

Acknowledgments

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Use of Phosphate-Blocking Groups in Ligase Joining of Oligodeoxyribonucleotides†

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ABSTRACT: The polynucleotide ligase from bacteriophage T_4 is able to join oligomers in which those terminal phosphate groups not directly involved in the formation of the new phosphodiester bond are in the form of alkyl phosphorothioates. These latter may be helpful in preventing alternate wron joinings and serving as a handle for subsequent fragmer modification.

One current strategy for the construction of deoxyribonucleotide duplexes ("genes") large enough and of the proper primary sequence to contain information that can, in principle, be transcribed into biologically nontrivial RNA involves the chemical synthesis of oligomers of sufficient size to be joined into larger arrays enzymatically (Agarwal *et al.*, 1970.)[1] This operation, catalyzed by polynucleotide ligase, requires that two segments to be joined must be held in adjace positions by separately associating, *via* conventional ant parallel Watson–Crick bonding, with a third fragment (t "splint") of appropriate complementary sequence so that t 3′-hydroxyl group of one (the "acceptor") is brought in close juxtaposition to the 5′-terminal phosphate of the oth (the "donor"). The splint thus provides specific templa guidance for the ligation proper.

There have been a few observations that *in vitro* joining m deviate from this scheme. Thus, it was found (Tsiapalis ar Narang, 1970) that the fidelity of the joining is not perfect; t ultimate base on the oligomer acceptor does not have to complementary to the corresponding counterbase on t splint. Furthermore, certain types f duplex "end-to-enc joining or terminal cross-linking were found to be complicati

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[1] Other approaches consist of the isolation of operons by genetic and physicochemical means (Shapiro *et al.*, 1969) or by "reverse" transcription of purified messengers (Ross *et al.*, 1972; Verma *et al.*, 1972; Kacian *et al.*, 1972).

EXHIBIT D

TABLE I: Oligodeoxyribonucleotides Used in Joining Reaction.[a]

Oligomer	Designation	Reference
d-EtS-p(T-G-C-T-A-A-A-T-T-T-G-A)	Protected fragment[2] 5	Heimer *et al.*, 1972a,b
d-p(A-A-G-A-C-A-G-C-A-T-A-T)	Fragment 2	Poonian *et al.*, 1972
d-EtS-p(T-G-T-C-T-T-T-C-A-A-A-T)	Protected fragment 3	This paper
d-EtS-p(T-T-A-A-T-C-C-A-T-A-T-G-C)	Protected fragment 1	Cook *et al.*, 1972
d-p(T-G-T-C-T-T-T-C-A-A-A-T)	Fragment 3	This paper
d-EtS-p(A-A-G-A-C-A-G-C-A-T-A-T)	Protected fragment 2	Poonian *et al.*, 1972

[a] See Discussion, Figure 2.

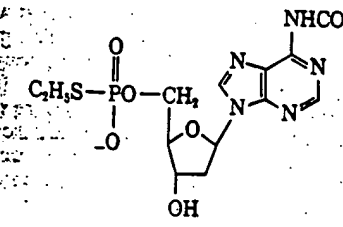
ents (Sgaramella *et al.*, 1970; Weiss, 1970). It was rea- oned that in order to minimize "wrong" joinings it would be seful t devise means whereby those terminal phosphates of he ternary nucleotide complex that could potentially result in n undesired phosphodiester linkage were prevented from oing s . We now find that it is possible in a ligase reaction to se both splint and acceptor molecules that have their 5′- erminal phosphates blocked with alkylthio groups—sub- tituents that can serve both as protecting groups during the emical oligonucleotide synthesis, and activating groups for ubsequent modification (Cook *et al.*, 1969). It is hoped that is extension of the substrate specificity of the enzyme will rove useful in the recovery of the valuable oligomer frag- ents not participating in the joining reaction.

xperimental Section

Enzymes. Polynucleotide kinase and polynucleotide ligase rom bacteriophage T_4 infected bacterial cells were the same as sed previously (Harvey and Wright, 1972). Calf intestinal ucosa alkaline phosphatase (type VII) was obtained from Sigma Chemical. The phosphatase was dialyzed against 0.01 Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$. The solu- ion was stored at a concentration of 1 mg/ml at −20°. No hosphodiesterase activity was found under the following con- itions: 10 nmol of the heptamer d-p(T-G-T-C-T-T-T)[2] was ncubated 1 hr at 37° with 20 μg of calf alkaline phosphatase. The dephosphorylated heptamer was labeled with ^{32}P using olynucleotide kinase and [γ-^{32}P]ATP as described above. The labeled heptamer was separated by DEAE-cellulose hromatography (Harvey *et al.*, 1971). The enzyme-treated eptamer was found to elute at the same position as the marker heptamer. This shows that no nucleotides were ex- sed by contaminating diesterases.

Bacterial alkaline phosphatase was obtained from Worth- ngton Biochemical and dialyzed against 0.01 M Tris-HCl uffer (pH 8.0). Pancreatic DNase (1 × crystallized), snake

[2] Nomenclature as specified in *Biochemistry* 9, 4022 (1970), and mplified in collaboration with Dr. W. E. Cohn. EtS preceding 5′- erminal phosphate symbol p denotes *S*-ethyl phosphorothioate. Thus, EtS-p(bzA) is



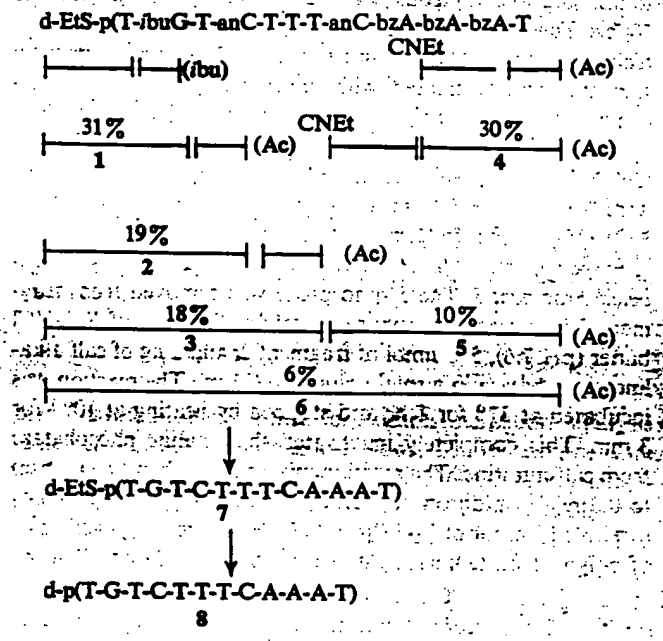


FIGURE 1: Chemical synthesis of fragment 3 (8) and its 5′-protected derivative 7.[3] Yields are given above the bars.

venom phosphodiesterase, spleen phosphodiesterase, and micrococcal nuclease were all purchased from Worthington Biochemical and used without further purification.

Oligonucleotides. The several oligodeoxyribonucleotides used in this study are summarized in Table I. In this paper we describe the synthesis of fragment 3 (Figure 1) and its 5′-protected derivative. General methods for oligonucleotide synthesis, including the procedure for condensation reactions, chromatographic techniques, analytical methods, and extinction values employed have been described in earlier papers. Figure 1 summarizes the synthetic approach[3]: the method of fragment condensation is employed. The outcome of individual steps is summarized under Results. Experimental details are given as legends to the figures. The 5′ terminus of the growing chain is carried as phosphorothioate ethyl ester throughout; its removal by mild oxidative hydrolysis constitutes the final chemical manipulation.

The dodecamer 7 was obtained by treatment of 6 with concentrated ammonium hydroxide overnight, followed by evaporation and chromatography on a Sephadex G-15 gel column (1 × 100 cm) which was eluted with 0.5 M triethylammonium

[3] The method of representation is patterned after peptide schemes; see, for instance, Rittel *et al.*, 1957.

fragment 5 ↓ fragment 2

(5′–3′)·····T GCT AAA TTT GAA AGA CAG CAT AT²· · · · · ·

(3′–5′)···· T AAA CTT TCT GTC GTA TAC CTA ATT

fragment 3 ↑ fragment 1

(5′–3′)·····U·GCU·AAA·UUU·GAA·AGA·CAG·CAU·AUG·GAU·UAA

-Ala - Lys - Phe - Glu - Arg - Glu - His - Met - Asp - ochre

FIGURE 2: Relati nship of DNA fragments t corresponding RNA and peptide sequence.

bicarbonate (pH 7.5). Unprotected dodecamer 8 was obtained by treatment of 6 (30 OD_{260} units) in phosphate buffer (pH 7), 0.4 M (0.25 ml), and water (0.5 ml) with a solution of iodine (0.2 M) in aqueous potassium iodide (0.4 M, 0.25 ml) for 18 hr. The product was diluted to 5 ml with water, and extracted with ether (three 5-ml portions). The aqueous portion was evaporated, treated with concentrated ammonium hydroxide (5 ml) overnight and centrifuged. The supernatant was evaporated and applied to an agarose gel column (Bio-Gel A, 0.5 m, 200–400 mesh, 0.85 × 115 cm) which was eluted with 0.5 M $Et_3NH_2CO_3$; fraction size, 4 ml, flow rate, 8 ml/hr; 8 (21 OD_{260} units, 75%) was located in fractions 17–20.

Preparation of 5′-³²P Labeled Fragment 2 and 5′-³²P Labeled Fragment 3. The 5′-phosphate was removed from fragment 2 in a reaction mixture containing 20 μmol of Tris-HCl buffer (pH 7.6), 5–8 nmol of fragment 2, and 5 μg of calf alkaline phosphatase in a total volume of 0.1 ml. The reaction was incubated at 37° for 1 hr and stopped by heating at 100° for 3 min. This completely inactivated the alkaline phosphatase from calf intestine. The reaction mixture was then brought up to 0.3 ml by addition of 3 μmol of $MgCl_2$, 2 μmol of dithiothreitol, 10 nmol of [γ-³²P]ATP (10 × 10^6 cpm), and 10 units of polynucleotide kinase. After incubation for 1 hr at 37°, the reaction mixture was separated on a Sephadex G-25 column (0.9 × 43 cm) by development with 0.5 M triet ammonium bicarbonate buffer (pH 7.6) at a flow rate of 6 hr, 0.45-ml fractions being taken. The ³²P-labeled fragme was found at the void volume, well separated from the pea [γ-³²P]ATP which followed. Fractions containing the des 5′-³²P labeled fragment 2 were concentrated *in vacuo* to ness and dissolved in 1 ml of H_2O.

5′-³²P labeling of fragment 3 was carried ut by the s procedure as described for fragment 2. [γ-³²P]ATP was p by the procedure of Weiss *et al.* (1968) except the ATP purified on a DEAE-cellulose column (0.9 × 15 cm) el with a linear gradient of 0–0.5 M triethylamm nium bi bonate buffer (pH 7.6) in a total volume of 400 ml.

Joining of Fragments and Separation of Products. The r tion mixture contained 66 mM Tris-HCl buffer (pH 7.6), mM $MgCl_2$, 6.6 mM dithiothreitol, 5 nmol of ATP, and 1 n of each strand (see Discussion and Figure 2) in a total vol of 0.06 ml. The reaction mixture was incubated at 0°. wi units of T_4 ligase. At 1-hr intervals, 1-μl samples were remc and the ³²P label assayed for resistance t bacterial alka phosphatase. The ³²P resistant to phosphatase reache maximum (40–60% in diester linkage) after 2 hr of incubat The entire reaction mixture was layered on an Agarose 0. column (0.9 × 62 cm). The column was developed at 6 m with 0.5 M triethylammonium bicarbonate and 0.45-ml tions were taken.

Assays for Phosphodiester Bond Formation. The assay for phosphodiester bond formation measured change of label from phosphatase labile to resistant. This was acc plished by incubation of the sample with 0.1 ml of 0.1 M HCl buffer (pH 8.0) and 5 μg of bacterial alkaline p

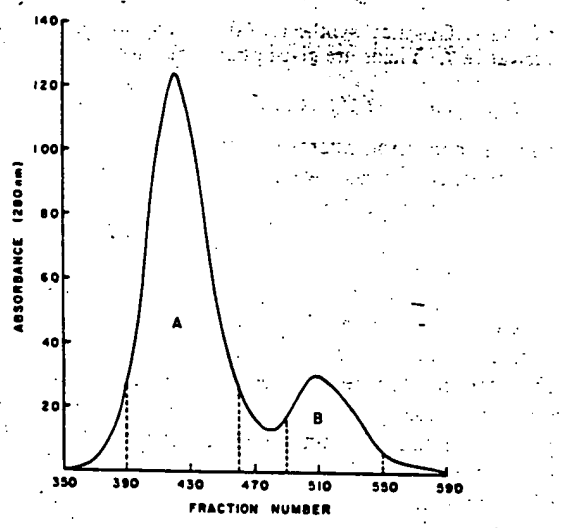


FIGURE 3: Preparation of 1. Condensation, 20 mmol of d-EtSpT (Cook *et al.*, 1972), 20 mmol of d-p*i*buG(*i*Bu), 45 mmol of $MesSO_2$-Cl (mesitylenesulfonyl chloride), 75 ml of pyridine, 3 hr. Work-up, 25 ml of water; after storage vernight at 0°, dilute to 200 ml with pyridine, treat with 200 ml of 2 N sodium hydroxide f r 5 min at 10°, neutralize with pyridinium Dowex 50, subject to preliminary DEAE-cellulose chromatography (not shown) with linear gradient of 0.3 M 20% ethanolic $Et_3NH_2CO_3$ buffer (pH 7.5) into 0.15 M 20% ethanolic $Et_3NH_2CO_3$ buffer. The material emerging with buffer molarity f 0.17–0.21 was c ncentrated and reapplied t a DEAE column (9.2 × 75 cm). Gradient, convex, 0.3 M 20% ethanolic $Et_3NH_2CO_3$ buffer pH 7.5 int 9 l. of 0.1 M 20% ethanolic buffer; flow rate, 2.5 ml/min; fraction size, 20 ml.

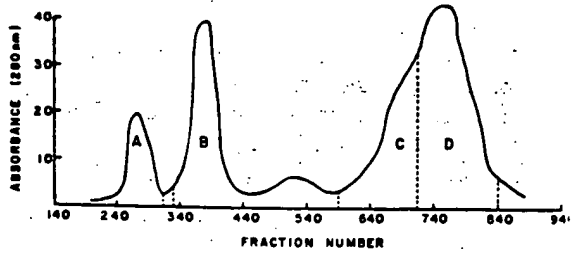
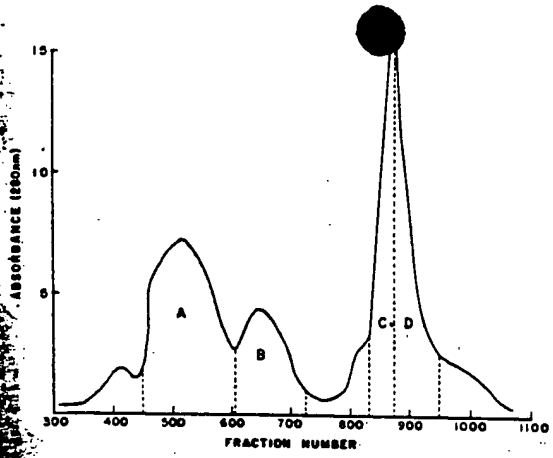


FIGURE 4: Preparation of 2. Condensation, 6.1 mmol f 1, 5 mm d-p(T-anC(Ac)) (Kumar and Khorana, 1969), 19 mm l of Mes Cl, 40 ml of pyridine, 3 hr. Work-up, 40 ml of water; after stc overnight at 5° adjust to 75 ml with pyridine and treat with 75 r 2 N NaOH for 20 min at 25°, neutralize with pyridinium Dowe Chr matography, DEAE-cellulose (6.6 × 90 cm), convex gra 0.275 M $Et_3NH_2CO_3$ pH 7.5 int 9 l. of 0.05 M buffer; fraction 20 ml; fl w rate, 2.5 ml/min. Fractions 720–840 were concentr and d-p(T-anC) was removed by gel permeati n chromatogr (not sh wn) on Sephadex G-25, superfine. Fractions 205 contained pure 2. Column size, 5 × 100 cm; fl w rate, 1 ml of buffer 0.2 M $Et_3NH_2CO_3$ pH 7.5; fraction size, 4 ml.



GURE 5: Preparation of 3. Condensation, 0.85 mmol of 2, 1.7 mmol of d-p(T-T-T(Ac)) (Narang *et al.*, 1969), 7.4 mmol of $MesSO_2Cl$, 30 ml of pyridine, 3 hr. Work-up, 30 ml of water; after storage overnight at 5° adjust to 100 ml with pyridine and treat with 100 ml of sodium hydroxide (2 N) at 0° for 10 min and neutralize with pyridinium Dowex 50. Chromatography, DEAE-cellulose (4.4 × 98 cm) bicarbonate form; gradient, linear, 12 l. of 0.45 M $Et_3NH_2CO_3$ pH 7.5 into 12 l. of 0.05 M buffer; flow rate, 2.5 ml/min; fraction size, 20 ml.

phatase for 30 min. After cooling, 0.2 ml of a solution containing 2 mM sodium pyrophosphate, 25 mM potassium phosphate buffer (pH 7.0), and 5 mg/ml of bovine albumin, followed by 0.2 ml of a 20% Norit suspension (packed volume) were added. The suspension was filtered through a 2.5-cm diameter glass fiber disk (Schleicher and Schuell). The residue was washed three times with cold 0.01 N HCl. The wet filter disk with washed Norit was placed in a vial with 10 ml of toluene-based scintillation fluid and ^{32}P determined in Packard scintillation spectrometer. Samples containing ^{33}P in place of ^{32}P as label were handled the same except the supernatant was counted and substracted from total radioactivity to determine the Norit adsorbable (or phosphatase resistant) label. This was necessary because of the lower energy of ^{33}P, which is counted in the ^{14}C channel.

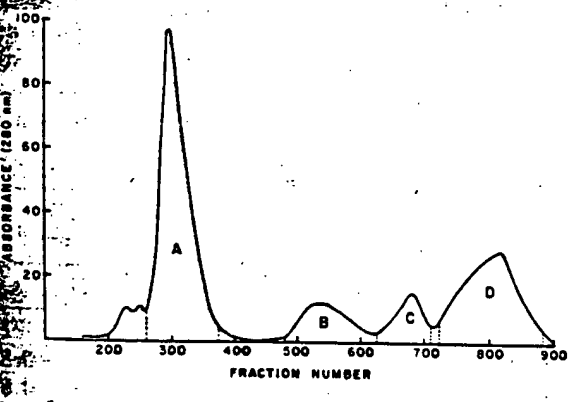


FIGURE 6: Preparation of 4. Condensation, 5 mmol of d-CNEt-p-(bzA-bzA) (Narang *et al.*, 1967), 20 mmol of d-pT (Ac), 35 mmol of $MesSO_2Cl$, 100 ml of pyridine, 3 hr. Work-up, 50 ml of water, 70 ml of iPr_2EtN; after storage overnight at 5°, dilute to 150 ml with pyridine and treat with 150 ml f 2 N sodium hydroxide at 0° for 20 min; neutralization with pyridinium Dowex 50. Chromatography, DEAE-cellulose, bicarbonate, (6.6 × 90 cm). Gradient, convex, 0.25 M $Et_3NH_2CO_3$ pH 7.5 into 0.05 M, 9 l.; flow rate, 2.5 ml/min; fraction size, 20 ml. Peak D was evaporated and acetylated using 25 ml f acetic anhydride in 40 ml f pyridine for 18 hr. After water addition (25 ml) the solution was evaporated and isolated by precipitation in the usual way.

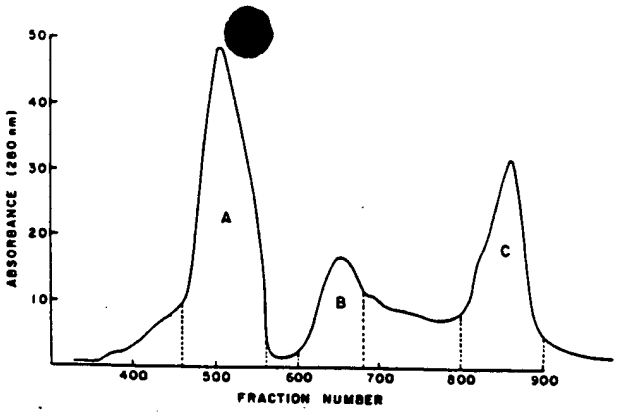


FIGURE 7: Preparation of 5. Condensation, 3.68 mmol of d-CNEt-p-(anC-bzA (Kumar and Khorana, 1969), 1.44 mmol of d-p(bzA-bzA-T(Ac), 9.15 mmol of $MesSO_2Cl$, 25 ml of pyridine, 2.5 hr. Work-up, 20 ml of water, 18 ml of iPr_2EtN, and storage overnight at 5°. After dilution to 50 ml with pyridine, treat with 50 ml of 2 N sodium hydroxide at 0° for 20 min and neutralize with pyridinium Dowex 50. Chromatography, DEAE-cellulose, bicarbonate (4.4 × 78 cm). Gradient, linear, 12 l. of 0.45 M $Et_3NH_2CO_3$ pH 7.5 into 12 l., 0.05 M; fraction size, 20 ml; flow rate, 2.5 ml/min. Peak C was evaporated and applied to a Sephadex G-50 (superfine) gel column (5 × 100 cm) (not shown) and eluted with 0.2 M $Et_3NH_2CO_3$; fraction size, 5 ml; flow rate, 1 ml/min. Pure d-p(anC-bzA-bzA-bzA-T) was obtained in fractions 234–250. The product was acetylated as described for 4.

Analysis for "Nearest Neighbor." The degradation of ^{32}P- or ^{33}P-labeled joined strands for "nearest neighbor" analysis (Josse *et al.*, 1969) was done as follows. The reaction mixture (0.2 ml) contained 4 OD_{260} calf thymus DNA, 50 mM triethylammonium bicarbonate buffer (pH 8.7), 2 mM $CaCl_2$, and approximately 10,000 cpm of ^{32}P- or ^{33}P-labeled joined strand. The mixture was incubated 4 hr with 60 units f micrococcal nuclease. After incubation, the mixture was adjusted to pH 5.0 with 1 N acetic acid (≈5 μl) and 2 μl of 1 M

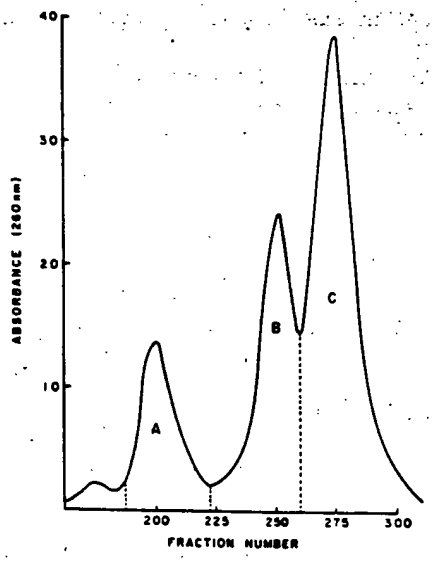


FIGURE 8: Preparation of 6. Condensation, 0.044 mmol of 3, 0.05 mmol of 5, 0.55 mmol of $MesSO_2Cl$, 2 ml of pyridine, 1.5 hr. Work-up, 1.5 ml of water, 0.5 ml of iPr_2EtN; after storage overnight at 5°, apply t a Sephadex G-50 (superfine) column (5 × 100 cm), elute with 0.2 M $Et_3NH_2CO_3$; fraction size, 3.8 ml; flow rate, 1 ml/min. Peak A was rechromatographed on a DEAE-cellulose column (2.5 × 85 cm) (not shown); gradient, linear, 8 l. of 0.4 M $Et_3NH_2CO_3$ into 8 l. of 0.3 M; fraction size, 20 ml; flow rate, 1 ml/min. Fractions 340–420 contained pure 6.

TABLE II: Identification of Chromatographic Peaks in Figures 3–8.

Figure	Peak A	Peak B	Peak C	Peak D
3	d-EtS-p(T-*i*buG)	d-p*i*buG		
4	$MesSO_3H$[a]	d-EtS-p(T-*i*buG)	d-p(T-anC)	d-EtS-p(T-*i*buG-T-anC) + d-p(T-anC)
5	d-p(T-T-T)	d-EtS-p(T-*i*buG-T-anC)	3 + impurity	Pure 3
6	dpT	Unknown	d-p(bzA-bzA)	d-p(bzA-bzA-T)
7	d-p(anC-bzA) + d-p(bzA-bzA-T)	Unknown	d-p(anC-bzA-bzA-bzA-T) + impurity	
8	6 + impurity	3	Mostly 5	

[a] $MesSO_3H$, mesitylenesulfonic acid.

TABLE III: Monomer Composition of Synthetic Intermediates of Fragment 3.

Intermediate	Mol % (Theory) A	C	G	T
d-EtS-p(T-G)			49.6 (50)	50.4 (50)
d-EtS-p(T-G-T-C)		25.2 (25)	24.4 (25)	50.4 (50)
d-EtS-p(T-G-T-C-T-T-T)		15.6 (14.3)	13.0 (14.3)	71.4 (71.4)
d-p(A-A-T)	63.8 (66.7)			36.2 (33.3)
d-p(C-A-A-A-T)	59.3 (60)	20.0 (20)		20.7 (20)
d-EtS-p(T-G-T-C-T-T-T-C-A-A-A-T)	25.1 (25)	17.0 (16.7)	6.5 (8.3)	51.4 (50)

potassium phosphate (pH 6.5) was added to inhibit phosphatases. The reaction was incubated with 1.65 units of spleen phosphodiesterase for 2 hr and stopped by heating to 100° for 2 min. The 3′-nucleotides were separated and examined for radioactivity.

Other Materials. The ATP used was obtained and purified as described earlier (Harvey and Wright, 1972). Crystalline bovine albumin was purchased from Schwarz–Mann. Agarose (Bio-Gel A, 0.5 m) was obtained from Bio-Rad Laboratories and columns prepared as recommended by the distributor.

Results and Discussion

The studies here detailed were carried out in connection with a wider synthetic program of molecules containing information for defined peptide sequences. Specifically, the segments joined by ligase here constitute the "right" end of a "gene" coding for a modified *S*-peptide of ribonuclease A (Finn *et al.*, 1968). Figure 2 depicts the relationships between the chemically synthesized DNA fragments consisting of an (upper) nonsense and (lower) sense strand, the corresponding RNA sequence, and the c gnate peptide chain. Chemical synthesis f deoxyribonucleotide oligomers is practical up to a point: when a size of 10–20 units is reached, enzymatic joining of such molecules becomes possible.

The synthesis of fragment 3 is shown in Figure 1. Figures 3–8 summarize the chromatographic purificati n of the reaction mixtures, and Table II identifies the peaks therein. The *S*-ethyl group was employed as the 5′-terminal blocking group; it was retained until completion of the chain anc moved using iodine–water (see Cook *et al.*, 1972, for fur details of this group). Oligonucleotides were analyzed (T. III) for their base content by ammonia hydrolysis followe snake venom diesterase digestion and high-pressure lic chromatography (Gabriel and Michalewsky, 1972). P chromatographic properties are summarized in Table IV.

TABLE IV: Paper Chromatography of Fragment 3 and Synth Intermediates.

Compound	M bility (dpT = for System[a] A	B	C
d-EtS-p(T-G)	0.98	1.18	1.2
d-EtS-p(T-G-T-C)	0.72	0.85	0.8
d-EtS-p(T-G-T-C-T-T-T)	0.36	0.52	0.6
d-p(A-A-T)	1.09	0.55	0.7
d-p(C-A-A-A-T)	0.96	0.30	0.5
d-EtS-p(T-G-T-C-T-T-T-C-A-A-A-T)	0.22		0.1
d-p(T-G-T-C-T-T-T-C-A-A-A-T)	0.18		0.0

[a] System A, isobutyric acid–concentrated ammoni hydroxide–water (57:4:39, v/v/v); B, ethanol–1 M ammoni acetate, pH 7 (1:1, v/v); C, 1-propanol–c ncentrated a monium hydroxide–water (55:10:35, v/v/v).

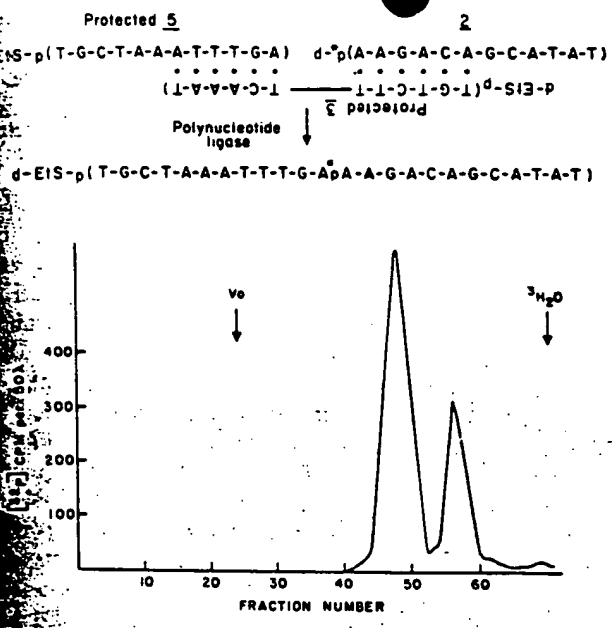


FIGURE 9: Separation of product after joining fragment 2 to protected fragment 5 in the presence of protected fragment 3. The ligase-joining reaction and details of separation by gel chromatography are given in the Experimental Section. The first peak to emerge is the joined material; the second represents the mixture of starting mate-

Two joinings were carried out with polynucleotide ligase from bacteriophage T_4: fragment 2 (Figure 2) was joined to the protected fragment 5 under the template guidance of protected fragment 3, and fragment 3 was in turn joined to protected fragment 1 under similar control of protected fragment 2. The reactions were monitored by prior labeling of the donor components (fragments 2 and 3) with tracer phosphate at the 5′ terminus, and their incorporation into molecules of greater size was observed in gel chromatography (Figures 9 and 10). As expected, the peaks emerging in front of the input donor molecules had the phosphate label in phosphatase-resistant phosphodiester linkage.

Nearest neighbor analysis proved the specificity of the indicated joinings: the radioactive 3′-nucleotide produced from the micrococcal spleen digest of the joining of fragment 2 to protected fragment 5 was exclusively [^{32}P]dAp, whereas, in the case of joining fragment 3 to protected fragment 1, [^{32}P]dCp was obtained.

There are several problems connected with such biochemical condensations: in addition to the possible complications mentioned in the introductory statement, the fact that the three components of such joining reactions are of similar size makes their recovery difficult. Here the presence of the phosphorothioate termini may be helpful; aside from the fact that they modify the chromatographic behavior of oligomers by their lipophilicity, and that they inherently carry one charge less than the corresponding primary phosphates, their reaction toward a large variety of nucleophiles (Cook *et al.*, 1969) makes subsequent modification—including reaction with macromolecular species—possible and thus may als be helpful in recovery attempts.

In summary, the substrate specificity of polynucleotide ligase from bacteriophage T_4 has been extended to oligomers carrying phosphorothioate termini at those sites n t involved in the generation of the new phosph diester bond.

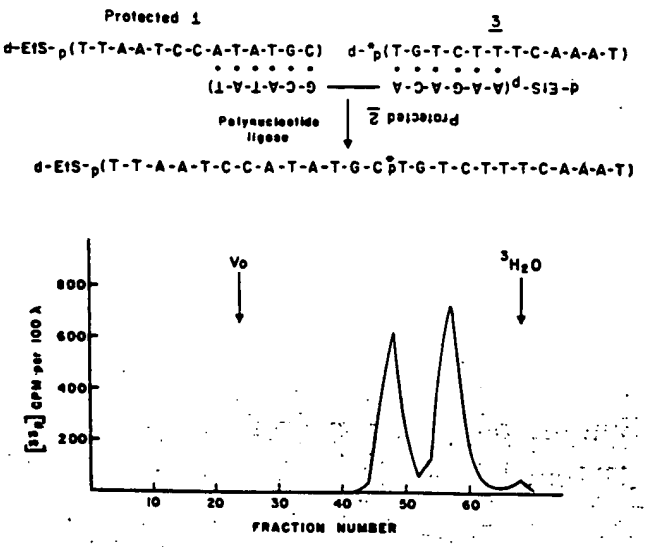


FIGURE 10: Separation of product after joining fragment 3 to protected fragment 1 in the presence of protected fragment 2. Details of joining reaction and gel chromatography are given in the Experimental Section. Peaks as in Figure 9.

Acknowledgments

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Binding of Ethidium Bromide to Double-Stranded Ribonucleic Acid†

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ABSTRACT: The interaction of ethidium bromide with double-stranded RNA (*Penicillium chrysogenum*) has been investigated using spectroscopic; spectropolarimetric, hydrodynamic, and thermal melting techniques. The binding isotherms (Scatchard plots) are dependent on ionic strength. The apparent binding constants and number of binding sites are quite similar to those found for DNA under similar conditions (Waring, M. J. (1965a), *J. Mol. Biol. 13*, 269). Hydrodynamic studies of the dye–RNA complex show a 53% increase in its viscosity increment, a 13% decrease in its relative sedimentation coefficient, and a decrease in its buoyant density in Cs_2SO_4 as compared to RNA alone. Thermal me studies show a marked increase in the T_m (ΔT_m = 26°). ible-region circular dichroic bands are induced when the is bound to RNA. These effects are also very similar to th sults of studies on ethidium bromide–DNA complexes (gleish, D. G., Peacoche, A. R., Fey, G., and Harvey, C. (1 *Biopolymers 10*, 1853; LePecq, J. B., and Paoletti, C. (1 *J. Mol. Biol. 27*, 87). Our data appear to indicate two mod binding of the dye to RNA which are consistent with ele static and intercalative interactions.

Ethidium bromide is a dye which has been widely used in nucleic acid binding studies. As a drug, it has trypanocidal, antibacterial, and antiviral activities (Dickenson *et al.*, 1953; Newton, 1964). The dye inhibits DNA polymerase (Elliott, 1963) and DNA-dependent RNA polymerase (Waring, 1964). *In vitro* the dye binds to both RNA and DNA (Waring, 1965a).

Two main modes of binding to native DNA have been suggested based on the results of spectral and hydrodynamic studies. The primary and generally stronger mode of binding has been interpreted as "intercalation" where a part of the ethidium ion sandwiches between adjacent base pairs. Spectral shifts in the 480-mμ absorption band of the dye (Waring, 1965a) together with a decrease in sedimentation coefficient (LePecq and Paoletti, 1967) and an increase in viscosity (LePecq, 1965) with extent of binding occurs on formation of the complex. The hydrodynamic changes, indicative of lengthening of the DNA polymer, support the intercalation hypothesis. A decrease in buoyant density upon binding of the dye to DNA has also been observed (LePecq and Paoletti, 1967).

Hydrodynamic changes also occur in closed circular DNA in the presence of ethidium bromide. These changes can be related to changes in superhelical density due to intercalation (Crawford and Waring, 1967; Bauer and Vinograd, 1968). Recent electron microscopic studies show a 27% increase in molecular length for a linear DNA–ethidium bromide complex and a relief of superhelical twisting in closed cir DNA in the presence of ethidium bromide (Freifelder, 1

The second and generally weaker mode of binding is evident at low salt and high ethidium bromide concentra This mode is thought to be an electrostatic interaction tween the phosphate groups in the double-stranded nu acid backbone and the dye molecules.

The same types of spectral effects have been observed v ethidium bromide binds to RNA. A number of RNAs o defined secondary and tertiary structure have been studie cluding ribosomal (Waring, 1965a), "core" (Waring, 19 and tRNA (Bittman, 1969). LePecq and Paoletti (1 postulated intercalative binding of ethidium bromide pr entially to helical regions in RNA. Waring (1965b) u spectral techniques studied binding of the dye to a grou synthetic polynucleotides. He was able to establish a rela ship between the degree of secondary (helical) structure the strength of primary binding. In these spectral studie RNA and RNA-like polynucleotides, primary binding is sidered synonymous with intercalation. H wever, this posal must be viewed with some reservation since there i supporting hydrodynamic evidence for these systems and spectral effects in themselves ar not sufficient to defi mode of binding.

In the present study th interaction of ethidium bror with native double-stranded RNA (ds-RNA),[1] having ondary and tertiary structural characteristics and hy

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[1] Abbreviation used is: ds-RNA, double-stranded RNA.

APPENDIX E

OLIGONUCLEOTIDIC COMPOUNDS. XLII.* SYNTHESIS OF THYMIDINEPHOSPHOROTHIOYL-($O^{3'} \rightarrow O^{5'}$)-THYMIDINEPHOSPHOROTHIOYL-($O^{3'} \rightarrow O^{5'}$)-THYMIDINE**

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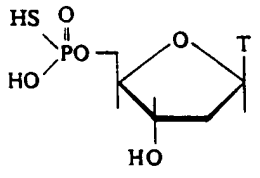
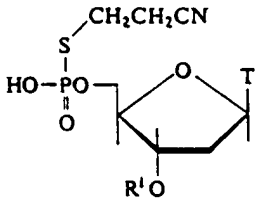
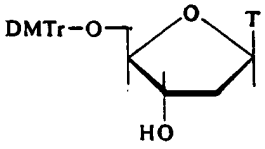
Reaction of thymidine 5'-phosphorothioate (*I*) with acrylonitrile at pH 8—9 and the subsequent treatment with acetic anhydride in pyridine affords 3'-O-acetylthymidine 5'-S-(2-cyanoethyl)-phosphorothioate (*IIb*). By the action of pyridinium S-(2-cyanoethyl)phosphorothioate and 2,3,5-triisopropylbenzenesulfonyl chloride, 5'-O-dimethoxytritylthymidine (*III*) is converted to 5'-O-dimethoxytritylthymidine 3'-S-(2-cyanoethyl)phosphorothioate (*IV*). Reaction of compounds *IIb* and *III* in the presence of 2,3,5-triisopropylbenzenesulfonyl chloride and the subsequent treatment with 90% aqueous acetic acid affords thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-3'-O-acetylthymidine [P-S-(2-cyanoethyl) ester] (*VI*). Reaction of compounds *IV* and *VI* accomplished by the action of 2,3,5-triisopropylbenzenesulfonyl chloride and the subsequent removal of protecting groups affords thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidine (*X*).



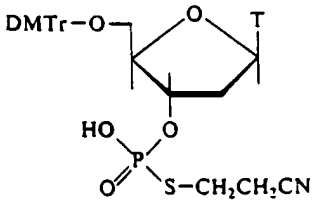
I



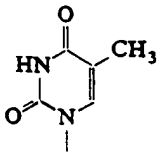
IIa, $R^1 = H$
IIb, $R^1 = COCH_3$



III



IV

In all formulae T = 

DMTr = dimethoxytrityl

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** Parts of this work have been reported in a preliminary communication[1].

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EXHIBIT E

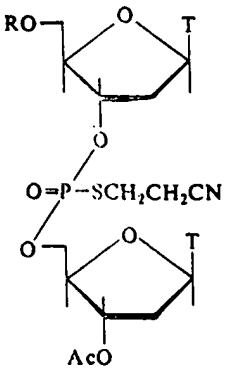
Polynucleotidic chains are used by the living matter for the storage of genetic informations as well as for the transport of these informations into the protein-synthesising systems. Processes involving informations stored in the sequence of bases in a polynucleotidic chain, may be investigated or controlled by means of synthetic chains of known sequences. The synthetic oligo- or polynucleotides may be successfully used only in simple biochemical systems lacking enzymes which cleave the internucleotidic bond. The more complex systems and the untouched living matter would probably require the use of such synthetic analogues that would be resistant towards enzymes or at least relatively less susceptible than the naturally occurring polynucleotides.

Such a requirement could be realised by synthesis of oligonucleotide analogues the internucleotidic bond of which is formed by phosphorothioic acid O,O-diester[2]. The enzymatically prepared polyribonucleotide analogues of this type maintain the ability to form double-stranded polymers and the messenger ability, being however more resistant towards nucleases than the parent substances.

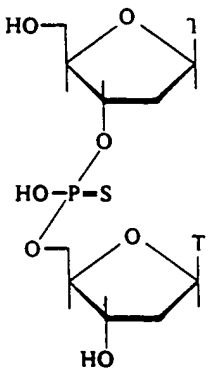
The O,O-dinucleoside esters of phosphorothioic acid have been synthesised by condensation of a nucleoside phosphorothioate with a nucleoside bearing a free hydroxylic function[3,4]; this method is not suitable for the preparation of longer chains since a mixture of O,O- and O,S-diester is obtained in each step. The unequivocal synthesis of phosphorothioic acid O,O-diester would to our opinion involve the O,O,S-triester; the sulfur atom of this triester would be protected by such a group which could be easily removed in the final step of the synthesis. This O,O,S-triester could be obtained by condensation of a O,S-diester with the hydroxylic function of the other component by the action of an aromatic sulfonyl chloride, analogously to the triester synthesis of the internucleotidic bond[5].

The realisation of this proposal has been first attempted in the deoxyribo series because of the easier accessibility of the starting compounds. The sulfur atom was protected by the 2-cyanoethyl group which has been some time ago proposed by Letsinger[5] for triester synthesis of the internucleotidic bond. The clue compound of the synthesis, namely, thymidine 5'-S-(2-cyanoethyl)phosphorothi ate (*IIa*) has been prepared by Cook[6] by reaction of thymidine 5'-phosphorothioate (*I*) with 3-bromopropionitrile. Alternatively, the S-(2-cyanoethyl) ester of nucleoside thiophosphates may be prepared by a direct cyanoethylation of nucleoside phosphorothioates with acrylonitrile[7]. Thymidine 3'-phosphorothioate is claimed[7] to react with acrylonitrile under buffered conditions to afford S-(2-cyanoethyl) ester along with 27% of the O-(2-cyanoethyl) ester (at pH 5·5) or with 13% of the O-ester (at pH 7·5). The amount of the O-ester in the crude product was however determined by a method which, to our opinion, was not suitable for this purpose. Thus, the crude cyanoethylation product was treated with potassium ferricyanide and then conc. aqueous ammonia was added after a certain period of time. The thus-obtained product was subjected after an inaccurately stated period of time to electrophoresis; the presence of the O-ester was deduced[7] from the formation of a bis(nucleoside-

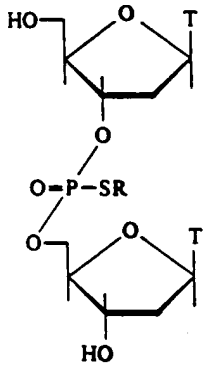
phosphoryl)disulfide with reference to the paper of Eckstein[8]; this author, however, performed the ferricyanide oxidation of phosphorothioic acid O-monoester, not of the



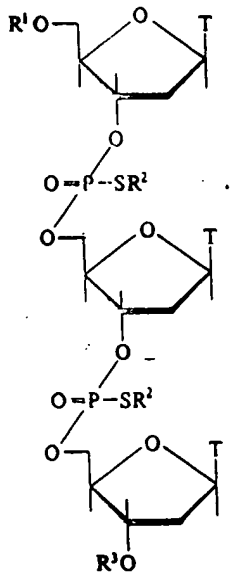
V, R = dimethoxytrityl
VI, R = H



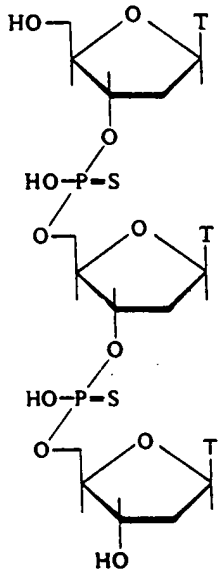
VIII



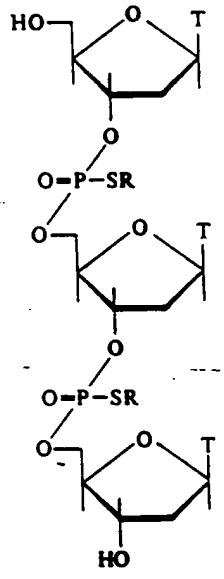
IXa, R = CH_2CH_3
IXb, R = $CH_2C_6H_4NO_2$



VIIa, R^1 = dimethoxytrityl, $R^2 = CH_2CH_2CN$, $R^3 = COCH_3$
VIIb, R^1 = H, $R^2 = CH_2CH_2CN$, $R^3 = COCH_3$



X



XIa, R = CH_3CH_3
XIb, R = $CH_2C_6H_4NO_2$

corresponding O,O-diester. The phosphorothioic acid O,O-diester can be hardly assumed to react with potassium ferricyanide since, *e.g.*, the bis(dialkoxyphosphoryl)-disulfides exhibit a higher oxidation-reduction potential than the ferricyanide[9]. In this laboratory, we did not observe any reaction of thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidine (*VIII*) with the ferricyanide. Addition of ammonia[7] to the ferricyanide-containing reaction mixture was accompanied by β-elimination of the 2-cyanoethyl group both with the O-(2-cyanoethyl) and the S-(2-cyanoethyl), ester, though at a somewhat slower rate in the latter case[6]. Consequently, the results depend on the time of action of the alkaline medium. We have shown that also the treatment of thymidine 5'-S-(2-cyanoethyl)phosphorothioate (*IIa*) with potassium ferricyanide and conc. aqueous ammonia led to the formation of the corresponding bis(nucleosidephosphoryl)disulfide (about 50% after 48 h).

In the cyanoethylation of phosphorothioic acid O-esters, the yields of the diesters were found to increase with the increasing pH value of the solution. At pH 8–9, the reaction is quantitative and affords exclusively the S-(2-cyanoethyl) derivatives under the conditions stated, as shown by iodine tests. When the O-(2-cyanoethyl) esters are formed at a lower pH value, they can be isomerised to the more stable S-isomers by raising the pH value[10].

According to Cook[6], thymidine S-(2-cyanoethyl)phosphorothioate (*IIa*) does not afford on self-condensation any oligomeric products; it could be inferred from this finding that the formation of an O,O,S-triester does not occur or that a 3',5'-cyclic triester is obtained. The reaction of 3'-O-acetylthymidine 5'-S-(2-cyanoethyl)phosphorothioate (*IIb*) with 5'-O-dimethoxytritylthymidine (*III*) by the action of 2,3,5-triisopropylbenzenesulfonyl chloride has been now observed to afford the O,O,S-triester *V* in 80% yield. By the action of aqueous ammonia, the triester *V* is converted to a dimethoxytrityl-containing substance, the immobility of which on thin-layer chromatography in 9 : 1 chloroform–methanol solvent system points to the occurrence of an ionic substance (O,O-diester). The O,O,S-triester *V* was also treated with 90% aqueous acetic acid and the course of detritylation was checked by thin-layer chromatography. The reaction was quantitative after 2 h. The product, namely, thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-3'-O-acetylthymidine [P-S-(2-cyanoethyl) ester] (*VI*), was isolated by chromatography on a loose layer of silica gel. Deblocking of the ester *VI* with 1 : 1 methanol-conc. aqueous ammonia afforded thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidine[1]. The above results have shown the realizability of the triester synthesis of phosphorothioic acid O,O-diester *via* O,O-dialkyl--S-(2-cyanoethyl) esters.

The above discussed synthesis was performed with the use of a nucleoside 5'-S--(2-cyanoethyl) ester. The other approach consists in the reaction of a nucleoside 3'-(2-cyanoethyl) ester with the $C_{(5')}$-hydroxylic function of the second component. In the latter approach, 5'-O-dimethoxytritylthymidine-3'-S-(2-cyanoethyl)ph sphorothioate (*IV*) served as the active component. Compound *IV* was prepared in a high

yield by condensation of 5'-O-dimethoxytritylthymidine (*III*) with the pyridinium salt of S-(2-cyanoethyl)phosphorothioate[11] by the action of 2,3,5-triisopropylbenzenesulfonyl chloride. This procedure represents the most advantageous method for the preparation of S-(2-cyanoethyl) derivatives of phosphorothioic acid O-esters as well as of phosphorothioic acid O-esters alone (because of the ready removability of the 2-cyanoethyl group), *cf.* ref.[10]

The phosphorothioate grouping of compound *IV* is resistant to potassium ferricyanide. In the presence of potassium ferricyanide, conc. aqueous ammonia splits off the 2-cyanoethyl group and the resulting phosphorothioic acid O-ester is oxidized with the ferricyanide unter the formation of the corresponding bis(nucleosidephosphoryl)disulfide. The 2-cyanoethyl and the dimethoxytrityl groups are quantitatively removed by the action of an acidic 1% solution of iodine in 50% aqueous acetone (checked by electrophoresis).

Condensation of the triester *VI* with the pyridinium salt of the diester *IV* by the action of triisopropylbenzenesulfonyl chloride afforded the protected trinucleotide *VIIa* in 38% yield. The dimethoxytrityl group was removed on treatment with 90% aqueous acetic acid under the formation of compound *VIIb*. Removal of the acetyl and the 2-cyanoethyl group from compound *VIIb* with the methanol-aqueous ammonia solvent mixture afforded the phosphorothioate analogue *X* of thymidylyl-thymidylyl-thymidine. The structure of compound *X* (as inferred from the synthesis) was confirmed on comparison with the thionucleotide *VIII* by chromatography (slower mobility of *X*) and electrophoresis (faster mobility of *X*). Another proof of structure of compounds *VIII* and *X* consists in S-alkylation with alkyl halides; this reaction is characteristic of salts of phosphorothioic acid O,O-diester[12,13]. Thus, treatment of compounds *VIII* and *X* with ethyl bromide in methanol and with *p*-nitrobenzyl bromide in dimethylformamide afforded products, the chromatographic and electrophoretic behaviour of which was similar to that of the O,O,S-triesters *IXab* and *XIab*.

The results of the present paper represent a starting point for investigations on the stepwise synthesis of analogues of oligonucleotidic chains carrying phosphorothioyl O,O-diester bonds.

EXPERIMENTAL

Thin-layer chromatography was performed on ready-for-use Silufol UV_{254} plates (Kavalier Glassworks, Votice, Czechoslovakia) in the following solvent systems: T_1, 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2); T_2, chloroform–methanol–pyridine (90 : 5 : 5); T_3, chloroform–methanol (9 : 1); T_4, chloroform–methanol–pyridine (8 : 1 : 1). The preparative runs were performed in the same systems on a 6 mm thick layer of loose silica gel (particle size, 10—60 micron) containing a fluorescent indicator (produced by Service Laboratories of this Institute in Prague - Suchdol). The dimethoxytrityl derivatives were detected by pressing a strip of paper to the m ist chromatographic layer of loose silica gel and spraying the paper with a 10% solution

of perchloric acid in 30% aqueous acetic acid. The bands were eluted with 1 : 1 chloroform–methanol solvent mixture (T_e). Electrophoresis was performed on paper Whatman No 1 (immersed in tetrachloromethane) in E_1, 0·05M triethylammonium hydrogen carbonate (pH 7·5).

Thin-layer chromatographical mobilities in systems T_1 and T_3, and the electrophoretical mobility in the buffer solution *E*: uridine 2′(3′)-phosphate (0·1, 0, 1·0); *I* (0·46, 0, 0·92); *II* (0·84, —, 0·50); *IV* (0·84, —, 0·05); *V* (—, 0·60, —); *VI* (—, 0·24, —); *VIIa* (—, 0·27, —); *VIIb* (—, 0·07, —); *VIII* (0·58, 0, 0·59); *XIa* (—, 0·16, —); *IXb* (—, 0·25, —); *X* (0·41, 0, 0·89); *XIa* (—, 0·15, —); *XIb* (—, 0·20, —).

Thymidine 5′-Phosphorothioate (*I*)

In the preparation of the title compound (*I*), the reported[6] procedure was used with s me modifications in the isolation. The reaction mixture consisting of 3′-O-acetylthymidine (2 mmol), S-(2-carbamoylethyl)phosphorothioate pyridinium salt (5 mmol), N,N′-dicyclohexylcarb diimide (2 g), pyridine (5 ml), and hexamethylphosphoric triamide is allowed to stand f r 4 days, diluted with water (5 ml), kept for additional 2 h, and evaporated under diminished pressure. The hexamethylphosphoric-triamide-containing residue is treated with 0·2M-NaOH (100 ml), the resulting mixture refluxed for 15 min, and allowed to cool. Pyridinium Dowex 50 ion exchange resin is then added to obtain pH 7. The resin is filtered off and the filtrate evaporated under diminished pressure. The residue is chromatographed on a 40 × 16 × 0·6 cm layer of loose silica gel in the solvent system T_1. The ultraviolet-absorbing band (R_F 0·55) is eluted with water and the eluate passed through Dowex 50 (H^+) ion exchange resin. The effluent is adjusted to pH 7·5 by the addition of barium hydroxide and concentrated under diminished pressure to the volume f 20 ml. The precipitate is removed by centrifugation and the supernatant is diluted with ethanol (40 ml). The solid is isolated by centrifugation, washed successively with 66% aqueous ethan l, 99% ethanol, and finally with ether, and air-dried. Yield, 454 mg of the barium salt of *I*.

3′-O-Acetylthymidine 5′-S-(2-Cyanoethyl)phosphorothioate (*IIb*)

The ammonium salt of compound *I* (obtained by the preparative thin-layer chr mat graphy as stated above) is dissolved in 50% aqueous dimethylformamide (8 ml) and the soluti n is adjusted to pH 8—9 with triethylamine. Acrylonitrile (2 ml) is then added, the whole mixture stirred at toom temperature for 20 h, and finally passed through a column of pyridinium Dowex 50 ion exchange resin (50 ml). The eluate was evaporated to dryness under diminished pressure and the residue coevaporated with three 10 ml portions of 9 : 1 ethanol–triethylamine. The ethanol is removed by coevaporation with pyridine and the final residue is dissolved in pyridine. As shown by spectrophotometry after chromatography of an aliquot on paper Whatman No 1 in the solvent system T_1, the solution contained 0·5 mmol of the triethylamonium salt of thymidine 5′-S-(2-cyanoethyl)phosphorothioate, identical on electrophoresis and chromatography with a specimen prepared according to ref.[6] As shown by quantitative decyanoethylation with iodine, the corresponding O-(2-cyanoethyl) derivative is absent.

Acetic anhydride (5 ml) is added to the above pyridine solution, the reaction mixture kept at room temperature for 20 h, and evaporated at 20°C/1 Torr. The residue is kept in 50% aqueous pyridine (10 ml) for 3 h and then passed through a column of pyridinium Dowex 50 (20 ml). The column is eluted with additional 50% aqueous pyridine and the eluate is evaporated at 20°C/1 Torr. The water is removed by repeated coevaporations with pyridine. The final residue is dissolved in pyridine (10 ml) and the solution is added dropwise with stirring into ether (300 ml). The precipitate is collected with suction, washed with ether, and dried under diminished pressure. Yield

279 mg of the pyridinium salt of compound *IIb*. For $C_{15}H_{20}N_3O_8PS.C_5H_5N$ (512·4) calculated: 10·94% N, 6·05% P, 6·25% S; found: 10·27% N, 6·00% P, 6·76% S.

5′-O-Dimethoxytritylthymidine 3′-S-(2-Cyanoethyl)phosphorothioate (*IV*)

A mixture of S-(2-cyanoethyl)phosphorothioate pyridinium salt[12] (2 mmol) and 5′-O-dimethoxytritylthymidine (1 mmol) is repeatedly coevaporated with pyridine at 20°C/1 Torr and the final residue is dissolved in pyridine (10 ml). The solution is shaken with 2,3,5-triisopropylbenzenesulfonyl chloride (600 mg) for 10 min, concentrated to a half of the original volume under diminished pressure, and the concentrate kept at 20°C for 20 h. Water is then added (5 ml) and the mixture is extracted with chloroform (30 ml). The extract is washed, dried over magnesium sulfate, concentrated to the volume of 10 ml, and the concentrate added dropwise with stirring into ether (200 ml). The precipitate is collected with suction, washed with ether, and dried under diminished pressure. Yield, 600 mg (78%) of the pyridinium salt of compound *IV*. For $C_{34}H_{36}N_3O_9PS.C_5H_5N$ (772·7) calculated: 7·25% N, 4·03% P, 4·19% S; found: 7·14% N, 3·85% P, 3·99% S.

5′-O-Dimethoxytritylthymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-3′-O-acetylthymidine [P-S-(2-Cyanoethyl)Ester] (*V*)

A mixture of the pyridinium salt of compound *IIb* (137 mg; 0·2 mmol) and 5′-O-dimethoxytritylthymidine (217 mg; 0·4 mmol) is coevaporated with three portions of pyridine and the final residue is shaken with 2,3,5-triisopropylbenzenesulfonyl chloride (180 mg) and pyridine (5 ml) for 10 min. The reaction mixture is concentrated just to crystallisation, kept at room temperature for 20 h, diluted with chloroform (3 ml), and chromatographed on one 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_2. The dimethoxytrityl-group-positive band (R_F 0·50) is eluted with the solvent system T_e, the eluate evaporated, and the residue dried under diminished pressure. Yield, 154 mg (80%) of the triester *V*. For $C_{46}H_{50}N_5O_{14}PS$ (959·9) calculated: 7·29% N, 3·23% P, 3·33% S; found: 6·98% N, 2·83% P, 3·17% S.

Thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-3′-O-acetylthymidine [P-S-(2-Cyanoethyl) Ester] (*VI*)

A solution of the triester *V* (130 mg) in 90% acetic acid (5 ml) is kept at 20°C for 2 h and evaporated at 20°C/1 Torr. The acetic acid is removed by repeated coevaporations with 1-butanol. The final residue is dissolved in chloroform and chromatographed on one 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_3. The ultraviolet-absorbing band (R_F 0·25) is eluted with the solvent system T_e, the eluate evaporated, and dried under diminished pressure. Yield, 80 mg f compound *VI*. For $C_{25}H_{32}N_5O_{12}PS$ (657·6) calculated: 10·66% N, 4·64% P, 4·87% S; found: 10·45% N, 4·39% P, 4·72% S.

5′-O-Dimethoxytritylthymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-3′-O-acetylthymidine [Bis-P_1-S,P_2-S-(2-cyanoethyl) Ester] (*VIIa*)

A mixture of the triester *VI* (60 mg) and the pyridinium salt of compound *IV* (155 mg) is coevaporated with three portions of pyridine and the residue is shaken with 2,3,5-triisopropylbenzenesulfonyl chloride (120 mg) in pyridine (5 ml) for 5 min. The reaction mixture is evaporated under diminished pressure just to crystallisation, kept at room temperature for 20 h, diluted with chloroform, and chromatographed on one 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_4. The dimethoxytrityl-group-positive band (9—15 cm) is eluted with the solvent system T_e, the eluate evaporated under diminished pressure, and the residue coevaporated re-

peatedly with toluene to remove pyridine. The residue is then rechromatographed as above except for the solvent system T_3. The ultraviolet-absorbing band (R_F 0·43) is eluted with the eluant T_e, the eluate evaporated, and the residue dried under diminished pressure. Yield, 44 mg (38%) of compound *VIIa*.

Thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidine (*X*)

A solution of compound *VIIa* (40 mg) in 90% aqueous acetic acid (5 ml) is kept at 20°C for 2 h, evaporated at 20°C/1 Torr, and the residue coevaporated repeatedly with 1-butanol to remove acetic acid. The thus-obtained detritylated derivative *VIIb* is dissolved in a mixture of methanol (1 ml) and conc. aqueous ammonia (1 ml), the solution kept at 50°C for 1 h, cooled down, and chromatographed on one 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_1. The ultraviolet-absorbing band (R_F 0·50) is eluted with water, the eluate evaporated to dryness under diminished pressure, the silicic-acid-containing residue taken up into a little water, filtered, and freeze-dried. Yield, 22 mg of the ammonium salt of compound *X*.

Reaction of O,O-Diesters *VIII* and *X* with Ethyl Bromide and *p*-Nitrobenzyl Bromide

Compounds *VIII* and *X* (about 1 mg each) were dissolved in 0·03 ml of methanol (reaction with ethyl bromide) or 0·03 ml of dimethylformamide (reaction with *p*-nitrobenzyl bromide), the solutions treated with the corresponding halide (about 10 mg each), the whole kept at 50°C for 6 h, cooled down, and chromatographed on a thin layer of silica gel (Silufol UV_{254}) in the solvent system T_3. On treatment with ethyl bromide, the O,O-diesters *VIII* and *X* were converted to the corresponding triesters *IXa* and *XIa* in c. 50% yield; the triesters *IXb* and *XIb* were obtained in an almost quantitative yield on treatment with *p*-nitrobenzyl bromide.

Reaction of Thiophosphoric Acid Diesters with Potassium Ferricyanide

A. Compounds *IIb* and *IV* (about 5 mg each) were kept with finely ground potassium ferricyanide (10 mg) in 50% aqueous acetone (0·05 ml) at 20°C for 20 h. As shown by chromatography in T_1 and electrophoresis in E_1, compound *II* did not react at all and compound *IV* split off the dimethoxytrityl group. The reaction mixtures were then treated with 0·05 ml of concd. aqueous ammonia each and subjected to electrophoresis in the buffer solution E_1. As shown by withdrawal of samples in intervals of 12 h, there are gradually formed electrophoretically more mobile compounds ($0{\cdot}84_{Up}$), namely, bis(nucleosidephosphoryl)disulfides. After 48 h, the yield is about 50%.

B. Powdered potassium ferricyanide (5 mg) was added to a solution of compound *VIII* (2 mg) in 50% aqueous acetone (0·05 ml). The samples were withdrawn in intervals of 24 h and analysed by chromatography in T_1 and electrophoresis in E_1. Even after 5 days, the starting compound *VIII* did not show any change.

Elemental analyses were carried out in the Analytical Department (Dr J. Horáček, Head) of this Institute.

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APPENDIX F

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Biochemistry

Selective inhibition of *Escherichia coli* protein synthesis and growth by nonionic oligonucleotides complementary to the 3′ end of 16S rRNA*

(oligonucleoside methylphosphonates/chemical synthesis/ribosome binding/cell-free translation/cell growth)

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ABSTRACT A series of nonionic oligonucleotide analogues, the deoxyribooligonucleoside methylphosphonates, were synthesized. The base sequences of these compounds, d(ApGpGp), d(ApGpGp)$_2$, and d[(ApGpGp)$_2$T], are complementary to the Shine–Dalgarno sequence (-A-C-C-U-C-C-U-) found at the 3′ end of bacterial 16S rRNA. These nonionic oligonucleotide analogues were tested for their ability to inhibit the *in vitro* translation of mRNAs in cell-free systems of *Escherichia coli* and rabbit reticulocyte. In the *E. coli* system, both d(ApGpGp)$_2$ and d[(ApGpGp)$_2$T] effectively inhibited MS-2 RNA-directed protein synthesis but they had much less effect on either poly(U)- or poly(A)-directed polypeptide synthesis. In the reticulocyte system, these compounds had no significant effect on the translation of globin mRNA. The observation that d[(ApGpGp)$_2$[^{3}H]T)] binds to 70S ribosomes (association constant, 2.0×10^4 M^{-1}, 37°C) together with the specificity of the inhibitory action of these compounds on pr tein synthesis strongly suggests that inhibition of translation is a consequence of analogue binding to Shine–Dalgarno sequence of 16S rRNA. The oligonucleoside methylphosphonates inhibited both protein synthesis (without concurrent inhibition of RNA synthesis) and colony formation by *E. coli* ML 308-225 (a permeable mutant) whose cell wall contains negligible quantities of lipopolysaccharide but had no effect on wild-type *E. coli* B. Our preliminary results on the uptake of oligodeoxyribonucleoside methylphosphonates by *E. coli* B show that these cells are not permeable t oligomers longer than 4 nucleotidyl units. Although oligodeoxyribonucleoside methylphosphonates are taken up by mammalian cells in culture, this series of analogues had negligible inhibitory effects on colony formation by transformed human cells. This study indicates that this class of nonionic oligonucleotide analogues can be used to probe and regulate the function and structure of nucleic acids of defined sequence within living cells.

Single-stranded exposed regions of cellular nucleic acids are pot ntial target regions for base-pairing interactions with complementary oligonucleotides. Binding of oligonucleotides to thes regions can be used to probe and regulate the structure–function relationship of nucleic acids in both biochemical and cellular systems. Deoxyribooligonucleotides complementary to the reiterated 3′- and 5′-terminal nucleotides of Rous sarcoma virus 35S RNA inhibited the translation of the RNA in a cell-free system as well as the virus production of chicken fibroblast tissue cultures (1, 2). Studies in our laboratory have shown that an oligonucleotide ethylphosph triester compl - mentary t the amin acid-accepting st m of most tRNAs had a transient but specific inhibit ry effect n the growth f mammalian cells in culture (3). More recently, we have studied th effects of oligo(dA) methylphosphonate analogues (compl m ntary t the anticodon loop of tRNA^{lys}) on bacterial and mammalian cells in culture (4). Th s anal gues contain an isost ric 3′-5′ linked methylphosphonate group which replaces the normal phosphodiester linkage of nucleic acids.

In this paper, we focus our attention on the 3′ end of 16S rRNA because the base-complementary interaction between the 3′-terminal polypyrimidine sequence -C-C-U-C-C-U- of 16S rRNA in the ribosome and the polypurine sequence -A-G-G-A-G-G- preceding the initiator triplet of mRNA is believed to be an essential recognition step in the initiation of protein synthesis in *Escherichia coli* (5). There have been several reports in support of this hypothesis (6–8). In comparison to the prokaryotic system, the 3′-terminal sequence of eukaryotic 18S rRNA differs from that of the 16S rRNA sequence (9) and, so far, there has been no concrete experimental evidence to suggest that initiation of eukaryotic protein synthesis involves a base-pairing mechanism similar to that of the prokaryotic system. This difference in the prokaryotic and eukaryotic systems led us to explore the possibility that protein synthesis in bacteria could be selectively inhibited by oligonucleotides complementary to the 3′ end of 16S rRNA. Inhibition of protein synthesis would be manifested by a reduction of colony formation by treated cells.

In order to exploit this possibility in living cells, we have synthesized a series of deoxyribooligonucleoside methylphosphonates with base sequences complementary to the 3′ end of 16S rRNA. Nonionic oligonucleoside methylphosphonates hav a number of unique physical and biochemical properties (10) including (*a*) the ability to form stable complexes with complementary polynucleotides, (*b*) the ability to penetrate the membranes of living cells, and (*c*) resistance to hydrolysis by cellular nucleases. In this paper, we report the synthesis of d[(ApGpGpApGpGp)T] and its intermediates and the effect of these analogues on cell-free protein synthesis in *E. coli* and rabbit reticulocyte systems as well as the effects of these analogues on *E. coli* B, *E. coli* ML 308-225 (a permeable mutant), and transformed human cells in culture.

MATERIALS AND METHODS

2′-Deoxyadenosine, 2′-deoxyguanosine, and thymidine were obtained from P-L Bioch micals and were checked for purity by paper chromatography before use. Poly(U) and poly(A) were purchased from Sigma. MS-2 RNA was a product of Miles. [^{3}H]Thymidin (101 Ci/mm l; 1 Ci = 3.7×10^{10} becqu rels), [^{3}H]lysin (54 Ci/mmol), [^{3}H]leucin (55 Ci/mmol), and

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Abbreviations: p, 3′–5′ linked methylphosphonate group. The symbols used to represent protected nucleosides and oligonucleoside methylphosphonates follow the IUPAC–IUB Commission on Biological Nomenclature Recommendations (1976).

* This is paper no. 4 of the series "Nonionic Oligonucleoside Methylphosphonates." Paper no. 3 is ref. 4.

[^{3}H]phenylalanin (35 Ci/mmol) were obtained from ICN. [^{3}H]Uridine (25 Ci/mmol), ^{3}H-labeled L-amino acid mixture, and the rabbit reticulocyt cell-free prot in synthesizing systems were purchased from New England Nuclear.

Preparation, purification, and is lation f deoxyriboolig nucleoside methylph sphonates were carried out as described (10). The bas ratios of the products were determined by depurination with 80% acetic acid (5 hr at 60°C). The resulting bases were separated by high-performance liquid chromatography on a reverse-phase Partisil ODS-2 column (Whatmann) with a 5–20% acetonitrile gradient in water (50 ml, total). Adenine and guanine had retention times of 5.6 and 2.0 min, respectively. Under the same conditions, d(ApGpGp) and d(ApGpGpApGpGp) had retention times of 12.5 and 18.0 min, respectively. The ratio of bases was determined from the area of the peaks. For d(ApGpGp) and d(ApGpGpApGpGp), the ratio of adenine to guanine was 1:1.9 and 1:1.95, respectively.

Dialysis experiments (11) were performed in 30-μl plexiglass chambers separated by a dialysis membrane. The equilibration buffer contained 60 mM Tris·HCl (pH 7.5), 120 mM NH_4Cl, 6 mM $MgCl_2$, 0.6 mM dithiothreitol, 0.6 mM GTP, 200 pmol of *E. coli* B ribosomes, and 135–175 pmol of ^{3}H-labeled deoxyribooligonucleoside methylphosphonates. The chambers were equilibrated at the desired temperature for 2 days before measurement.

A cell-free protein-synthesizing system and 70S ribosomes from *E. coli* B were prepared according to the method of Nirenberg (12). Cell-free protein synthesis in a rabbit reticulocyte system was performed by using a cell-free translation system purchased from New England Nuclear (lot J1157AW). For the translation of globin mRNA, the reactions were run in 25.0 μl of buffer containing 2 μl of translation mixture, 2 μg of globin mRNA, 79 mM potassium acetate, 0.65 mM magnesium acetate, 0–100 μM oligomer, and 14 μM [^{3}H]leucine. Reactions were initiated by addition of 5 μl of reticulocyte lysate. Aliquots (4 μl) were removed at various times and added to 0.1 ml of bovine serum albumin (100 μg) solution. The protein was precipitated by heating with 1 ml of 10% trichloroacetic acid at 70°C filtered on G/F filters, and assayed for radioactivity in Betafluor.

E. coli ML 308-225 cells (a gift from Chien Ho, Carnegie–Mellon University, Pittsburgh) were grown at 37°C in minimal salt medium supplemented with 1% glucose (13). *E. coli* B cells were grown in M9 medium as described (14). Protein synthesis and RNA synthesis were carried out in cells grown to midlogarithmic phase ($\approx 5.0 \times 10^8$ cells per ml). Aliquots (50 μl) of cells were preincubated with 15 μl of medium or medium containing the compounds for 1–2 hr at 22°C. *E. coli* ML cells were transferred to a water bath maintained at 10°C. After 10 min, 3 μl of [^{3}H]uridine (100 μCi/ml) or 3 μl of [^{3}H]leucine (50 μCi/ml) was added; then 15-μl aliquots were withdrawn at 0, 5, 10, and 20 min and added to 200 μl of lysing buffer (2.0% NaDodSO$_4$/0.02 M EDTA) and heated at 70°C for 20 min. For protein synthesis experiments, bovine serum albumin (100 μg) and 20% trichloroacetic acid (1 ml) were added and th soluti n was heated at 70°C for 15 min. Then the solution was filt red; the filter was then washed and assayed for radioactivity. For RNA synth sis experiments, cold 5% trichloroacetic acid was added after lysis f the cells, and the solution was filtered without h ating. Th final concentration of oligomers in these xperiments was 100 μM.

For determination f col ny formati n, *E. coli* ML 308-225 cells were incubated for 2 hr in 100 μl of medium containing 75–160 μM of oligonucleoside methylphosphonate. The s lution was then diluted to 1.0 ml with the medium. To 0.9 ml of this solution, 2.0 ml of 0.5% bactoagar was added at 37°C and the solution was poured onto 100-mm plates containing 1.5% bactoagar. After solidification, the plates were incubated at 37°C for 36 hr, and the colonies were counted. The final concentration of the oligomers on the plate was 2.6–5.5 μM.

In vitro aminoacylation experiments were done as described by Barrett *et al.* (15).

Growth experiments were done by treating 15 μl of cells ($\approx 1 \times 10^8$ cells per ml) in 15 μl of medium (control) or medium containing 150 μM of the compounds at 37°C. Aliquots (4 μl) were withdrawn at different time intervals and appropriat ly diluted. The number of cells was determined by using a Hausser counting chamber and a Zeiss phase-contrast microscope.

RESULTS

Table 1 summarizes the reaction conditions and yi lds in the preparation of the deoxyribooligonucleoside methylphosphonates. Because the trinucleotide sequence d(A-G-G) is repeated in the heptamer, condensation of the trinucleotide blocks was considered to be more favorable than the stepwise addition of mononucleotides. The fully protected heptamer was prepared by condensing T(OAC) or [^{3}H]T(OAC) with the protected hexamer (data not shown). The low yields obtained in these preparations are attributed to the large number of dG residu s present in these sequences. The trimer d(ApGpGp) and hexamer d(ApGpGpApGpGp) were deblocked fr m d([(MeO)$_2$Tr]bzApibuGpibuGpCNEt) and d([MeO)$_2$Tr]-bzApibuGpibuGpbzApibuGpibuGpCNEt), respectiv ly, and hence obtained with the 5′-terminal methylphosphonate group. Reactions carried out on a small scale (<0.01 mmol) w re deblocked as such and the product was isolated by paper chromatography. The purity of the oligomers was examined mainly by high-performance liquid chromatography and paper chromatography. The UV spectral properties and paper chromatographic mobilities are given in Table 2.

The interaction of d(ApGpGpApGpGp[^{3}H]T) and d(ApGpGp[^{3}H]T) with 70S ribosomes was studied by equilibrium dialysis. The heptamer has a high apparent associati n constant which decreases with increasing temperature (4.67×10^5 M^{-1} at 0°C; 1.72×10^5 M^{-1} at 22°C; 2.0×10^4 M^{-1} at 37°C). As expected, the tetramer, which has only three bases complementary to the 3′ end of 16S rRNA, has a proporti nately lower association constant (1.44×10^4 M^{-1} at 22°C).

Table 1. Preparation of protected oligodeoxyribonucleoside methylphosphonates

3′-Methylphosphonate		5′-OH		MST	Product		
						Yield	
Component	mmol	Component	mmol	mmol	Name	mmol	%
d([(MeO)$_2$Tr]ibuGp)	2.77	d(ibuGpCNEt)	2.99	6.64	d(ibuGpibuGpCNEt)	0.75	27
d([(MeO)$_2$Tr]bzAp)	1.1	d(ibuGpibuGpCNEt)	0.725	2.2	d([(MeO)$_2$Tr]bzApibuGpibu-GpCNEt)	0.15	21
d([(MeO)$_2$Tr]bzAp-ibuGpibuGp)	0.033	d(bzApibuG-pibuGpCNEt)	0.04	0.132	d([MeO)$_2$Tr]bzApibuGpibu-GpbzApibuGpibuGpCNEt)	0.01	30

Table 2. UV spectra and chromatographic mobilities of oligodeoxyribonucleoside methylphosphonates

Oligomer	UV spectra* λ max., nm	λ min., nm	$\varepsilon_{260/280}$	ε_{max}‡	Paper chromatography,† R_F
d(ApGpGp)	257	228	2.11	—	0.77
d(ApGpGpT)	257	229	2.02	4.19×10^4	0.88
d(ApGpGpApGpGp)	257	229	2.11	6.6×10^4	0.27
d(ApGpGpApGpGpT)	257	232	2.06	7.33×10^4	0.39

* In water at pH 7.0.
† Run in solvent F; R_F of pT, 0.41. Solvent F is *n*-propanol/NH_4OH/H_2O, 55:10:35 (vol/vol).
‡ Obtained by comparing the absorbance of a solution of the oligomer in water at pH 7.0 to that of the same solution at pH 1.0. The oligomer extinction coefficient was calculated from the observed hyperchromicity of the oligomer at pH 1.0 by using the following extinction coefficients: dA at pH 1.0, 14.1 × 10^3; dG at pH 1.0, 12.3 × 10^3.

The effects of the oligomers on cell-free protein synthesis in *E. coli* B system are summarized in Table 3. In general, the hexamer and heptamer exhibited inhibitory activities but the trimer and tetramer did not. Poly(U)-directed polyphenylalanine synthesis and poly(A)-directed polylysine synthesis were not inhibited appreciably by hexamer and heptamer at 37°C. The inhibition was greater at 22°C than at 37°C. At higher concentrations, the hexamer inhibited polylysine synthesis directed by poly(A) more effectively than polyphenylalanine synthesis directed by poly(U). Whereas d(ApGpGp) and d(ApGpGpT) did not cause appreciable inhibition of the translation of MS-2 RNA in the *E. coli* system, d(ApGpGpApGpGp) and d(ApGpGpApGpGpT) were effective inhibitors in dose-dependent manner, even at low concentrations. As a negative control for sequence specificity, d(CpCpApApGpCp-chlorophenylphosphate), a hexamer not complementary to the 3′ end of 16S rRNA, was used. This oligomer was found to be much less effective in inhibiting translation of MS-2 RNA in the *E. coli* system. In contrast to their effects on the *E. coli* system, both d(ApGpGpApGpGp) and d(ApGpGpApGpGpT), which are not complementary to the 3′ end of eukaryotic 18 S rRNA, did not have appreciable inhibitory effects on the translation of globin mRNA in a cell-free reticulocyte system (at 100 μM and 22°C, 16% and 17%, respectively).

The effects of deoxyribooligonucleoside methylphosphonates on the colony formation by *E. coli* B, *E. coli* ML 308-225, and transformed human cells (HTB 1080) as well as the effects of these analogues on cellular protein synthesis in *E. coli* B and *E. coli* ML-308-225 were investigated. Oligomers d(ApGpGp), d(ApGpGpApGpGp), and d(ApGpGpApGpGpT) inhibited colony formation by *E. coli* ML 308-225 cells effectively (Table 4).

Table 3. Effect of deoxyribooligonucleoside methylphosphonates on cell-free protein-synthesizing system from *E. coli* B

Oligomer	Conc., μM	Inhibition, % Poly(U)* 22°C	Poly(U)* 37°C	Poly(A)† 22°C	Poly(A)† 37°C	MS-2 RNA 22°C
d(ApGpGp)	100	8	0	0	0	5
d(ApGpGpT)	100	—	—	—	—	0
d(ApGpGpApGpGp)	12.5	—	—	—	—	45
	25	0	0	0	0	75
	50	19	0	29	14	88
	100	39	18	80	27	—
d(ApGpGpApGpGpT)	25	0	0	0	0	77
d(CpCpApApGpCp)‡	100	—	—	—	—	21

* At 260 μM in UMP residues.
† At 225 μM in AMP residues.
‡ p = *p*-chlorophenylphosphate.

These analogues had virtually no effect on colony formation by *E. coli* B cells and only a small inhibitory effect on colony formation by transformed human cells.

Results of the study on cellular protein synthesis by *E. coli* ML 308-225 support the observation on the colony formation by these two strains of bacteria. The rates of incorporation, by *E. coli* ML 308-225 cells, of exogenous [^{3}H]leucine into hot trichloroacetic acid-precipitable material and of [^{3}H]uridine into cold trichloroacetic acid-precipitable material were found to be quite rapid at 37°C and 22°C; however, the incorporation leveled off in 5 min at these temperatures. Hence, incorporation of [^{3}H]leucine and [^{3}H]uridine by this *E. coli* mutant were studied at a lower temperature (10°C). The incorporation was linear up to 10 min at this temperature. d(ApGpGpApGpGpT) inhibited protein synthesis by *E. coli* ML 308-225 but not by *E. coli* B. Some variation in the extent of inhibition was observed between experiments, and the inhibition was found to be in the range of 20–45%. Under the same experimental conditions, d(ApGpGpApGpGpT) had no effect on RNA synthesis as measured by [^{3}H]uridine incorporation. d(ApGpGpT) has no effect on either protein synthesis (Table 4) or RNA synthesis (data not shown).

In addition to the studies on colony formation, experiments were done on the growth of *E. coli* ML 308-225 in mass culture in the presence of the oligonucleotide analogues. There was no inhibition of growth during the first 4 hr (Fig 1). At the rapid growth period, between 4 and 12 hr, the growth of the treated culture was inhibited up to 50% in the presence of either trimer or heptamer. At the end of this growth period, 24 hr after initiation of the culture, the treated and untreated cultures had approximately the same number of cells.

DISCUSSION

The oligodeoxyribonucleoside methylphosphonates used in this study were prepared by the general procedures reported earlier

Table 4. Effect of deoxyribooligonucleoside methylphosphonates on colony formation

Oligomer (at 75 μM)	Inhibition, % *E. coli* ML 308-225*	*E. coli* B*	Human cells HTB 1080†
d(ApGpGp)	75–98	0	—
d(ApGpGpApGpGp)	78–97	0	—
d(ApGpGpApGpGpT)	67–97	0	10
d(ApGpGpT)	0	0	—
d(GpGpT)	5	—	—

* At either 22°C or 37°C.
† At 37°C.

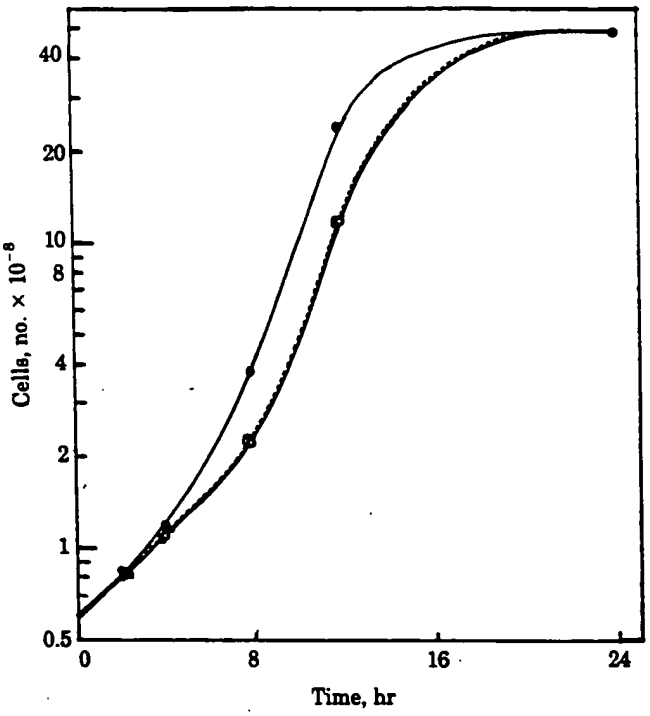

FIG. 1. Effect of oligonucleoside methylphosphonates on *E. coli* ML 308-225 cells growing in culture. ●, Control; ○, in the presence of 150 μM d(ApGpGp); □—□, in the presence of 150 μM d[(ApGpGp)$_2$T].

(10). The yields in these preparations are somewhat low, which can be attributed to the reactions involving deoxyguanosine residues. Similar difficulties have been encountered in the preparation of oligonucleotide phosphotriesters containing deoxyguanosine residues (16).

The two phosphonate analogues, d(ApGpGp[^{3}H]T) and d(ApGpGpApGpGp[^{3}H]T), which are complementary to the 3′ end of 16S rRNA, exhibit high affinity for 70S ribosomes as studied by equilibrium dialysis. Earlier studies on the interaction of a pentanucleotide (G-A-dG-dG-U) with *E. coli* 30S and 70S ribosomes have shown that this pentamer specifically binds to a site on the 30S subunit (7). Hence, it is very likely that the observed binding of the tetramer and heptamer analogues with 70S ribosomes is due to their formation of complexes with the complementary regions at the 3′ end of 16S rRNA.

This conclusion is further supported by studies on the effect of these analogues on cell-free protein synthesis. Both the hexamer and heptamer effectively inhibit the translation of MS-2-RNA in the *E. coli* cell-free system while having a much lower inhibitory effect on the translation of poly(U) and poly(A). The *in vitro* aminoacylation of $tRNA_{coli}$ is not inhibited by these oligomers, suggesting that the inhibition of aminoacylation of tRNAs does not play a role in the inhibition of translation of MS-2 RNA. The inhibition observed in the *E. coli* system, therefore, is most likely at the ribosome site. Because the synthetic mRNAs, unlike natural mRNAs, lack specific initiation sites, the results also support the conclusion that the inhibition of translation of MS-2 RNA may arise from competition between the oligonucleotide analogues and the homologous sequence within the preinitiator region of MS-2 RNA. Our results are in agreement with those obtained by Taniguchi and Weissmann (6) and Eckhardt and Luhrmann (7). They observed an inhibition of formation of phage mRNA-70S ribosome initiation complex in the presence of oligonucleotides complementary to the 3′ end of 16S rRNA. In contrast, no inhibition of poly(U)-dependent $tRNA^{Phe}$ binding to 70S ribosomes was found. Additional support for the base-complementary interaction of oligonucleotide analogues with the 3′ end of 16S rRNA comes from the inability of these analogues to inhibit the translation of globin mRNA in a rabbit reticulocyte system. Although the 3′-end sequences of 18S rRNA and 16S rRNA are similar, 18S rRNA specifically lacks the -C-C-U-C-C-U- sequence found in 16S rRNA, and hence the oligonucleotide analogues cannot form stable complexes with 18S rRNA in reticulocyte ribosomes.

Although the oligomers inhibit translation of mRNAs in the *E. coli* B cell-free system, these oligomers have no effect on either protein synthesis or colony formation by the intact *E. coli* B cells; however, as shown in Table 4, these oligomers inhibit the protein synthesis and growth of an *E. coli* mutant (ML 308-225). Our experiments on the uptake of oligonucleotides by *E. coli* B cells indicate that they are permeable to d(Ap[^{3}H]T), Tp[^{3}H]T, and TpTp[^{3}H]T but not to $(Tp)_4$[^{3}H]T and $(Tp)_8$[^{3}H]T (unpublished data). Thus, oligonucleoside methylphosphonates longer than 4 nucleotide units cannot enter the cell. The cutoff size of the nonionic oligonucleotides observed here agrees with the size limit found for oligosaccharides and oligopeptides (17, 18). In contrast to *E. coli* B, *E. coli* ML 308-225 cells were permeable to d(ApGpGpApGpGp[^{3}H]T) (unpublished data). This *E. coli* mutant has only small quantities of lipopolysaccharide in the outer membrane of the cell wall (19). The reduction in lipopolysaccharide content may increase the permeability of cell wall toward oligonucleoside methylphosphonates. Thus, the difference in the permeability of the cell walls of these two bacteria can explain why the hexamer and heptamer do not have any effect on intact *E. coli* B cells but inhibit protein synthesis, colony formation, and culture growth of *E. coli* ML 308-225.

The specific inhibitory effects of oligonucleotide analogues in the cell-free systems is also indicated by the following observations at the intact cellular level (Table 4). (*i*) The oligonucleotide analogues inhibit protein synthesis and growth of *E. coli* ML 308-225 cells but have little or no effect on human cells. (*ii*) Although d(ApGpGpApGpGp) and d(ApGpGpApGpGpT inhibit colony formation by *E. coli* ML 308-225, d(ApGpGpT has no effect; and (*iii*) d(ApGpGpApGpGpT) inhibits protein synthesis without concurrent inhibition of RNA synthesis.

Because we have found that oligonucleoside methylphosphonates $(Tp)_nT$ (n = 1,4,8) are effectively taken up by Syrian hamster cells (unpublished results), the lack of inhibition of human cell colony formation is unlikely to be attributable to the inability of these oligomers to penetrate the human cells. At present, the observed inhibitory effect of d(ApGpGp) on colony formation by *E. coli* ML 308-225 cells requires additional investigation for an adequate explanation because the trimer has no effect on cellular protein and RNA synthesis in this mutant. Also, the trimer has no effect on protein synthesis in the *E. coli* B cell-free system.

The temporary inhibition of the growth of *E. coli* ML 308-225 cells in mass culture by the trimer and heptamer (Fig. 1) is in agreement with the expectation that the oligomers may not have caused a permanent damage to the functioning of ribosomes. The cells can overcome this inhibition by synthesizing more ribosomes, or the inhibitory effect of the oligomers will not be detectable when the capacity of the ribosomes for protein synthesis is no longer the factor limiting growth.

The results of our studies further demonstrate the feasibility of using oligonucleoside methylphosphonate analogues for probing the structure–function relationship of nucleic acids in biochemical systems as well as in living cells. The results also suggest that, by choosing an appropriate complementary sequence of oligonucleotide, one can selectively regulate either

bacterial or mammalian cellular n[illegible]c acid function. Thus, the appropriate use of nonionic nucleic acid anal gues, such as the oligonucleoside methylphosph nates, may have great significance in basic research and in practical applications.

We thank Dr. Chien Ho and Mrs. Cottam for providing us with *E. coli* ML 308-225 cells and Cathy Alden and Dorothy Lindstrom for preparation of the manuscript. This research was supported in part by a grant from the National Institutes of Health (GM 166066-12).

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APPENDIX G

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARK
Washingt n, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
07/355,148	12/20/90	Tullis	P31-0756

EXAMINER	
J. Martinell	

ART UNIT	PAPER NUMBER
1805	12

DATE MAILED:

EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) Ms. Campbell (3) _____

(2) Exr. Martinell (4) _____

Date of interview 06/18/92 6/22 6/19/92

Type: ☐ Telephonic ☒ Personal (copy is given to ☐ applicant ☒ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☒ No. If yes, brief description: _____

Agreement ☐ was reached with respect to some or all of the claims in question. ☒ was not reached.

Claims discussed: All.

Identification of prior art discussed: All.

Description of the general nature of what was agreed to if an agreement was reached, or any other comments:

(a) Applicant intends to file copies of declaration from parent application to overcome 102(e)/103 rejection. (b) Applicant asserted that Exr. had indicated claims to be free of the Patterson et al 103 rejection during prosecution of the parent application and that the claims in the parent application were at that time not limited to phosphotriesters. (c) Exr. indicated that evidence in form of declaration(s) or references would strengthen any arguments in connection with the §112 rejections.

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must b attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

Unless the paragraphs below have been checked to indicate to the contrary, A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION I NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW (e.g., items 1—7 on the reverse side of this form). If a response to th last Office action has already been filed, then applicant is given one month from this interview date to provide a statement of the substance of the intervie

☐ It is not necessary for applicant to provide a separate record of the substance of the interview.

☐ Since the examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections an requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill th response requirements of the last Office action.

Examiner's Signature

PTOL-413 (REV. 1-84)

EXHIBIT G

APPENDIX H

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
06/314,124	10/23/81	TULLIS R	22178

FULWIDER, PATTON, RIEBER, LEE & UTECHT
3415 WILSHIRE BLVD., STE. 2400
LOS ANGELES, CA 90010

EXAMINER	
MARTINELL, J.	
ART UNIT	PAPER NUMBER
127	

DATE MAILED: 05/08/85

RECEIVED
MAY 10 1985
FULWIDER, PATTON, RIEBER, LEE & UTECHT

Molecular Biosystems GGK/CAC

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

Due: August 8, 1985

☒ This application has been examined ☒ Responsive to communication filed on 12/7/84 ☒ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), ~~days~~ from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892.
2. ☐ Notice re Patent Drawing, PTO-948.
3. ☒ Notice of Art Cited by Applicant, PTO-1449
4. ☐ Notice of Informal Patent Application, Form PTO-152
5. ☐ Information on How to Effect Drawing Changes, PTO-1474
6. ☐ _____

Part II SUMMARY OF ACTION

1. ☒ Claims 1, 3-28, and 30-53 are pending in the application.
   Of the above, claims 40-52 are withdrawn from consideration.
2. ☒ Claims 2, 29 have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☒ Claims 1, 3-28, 30-39, and 53 are rejected.
5. ☐ Claims _____ are objected to.
6. ☐ Claims _____ are subject to restriction or election requirement.
7. ☐ This application has been filed with informal drawings which are acceptable for examination purposes until such time as allowable subject matter is indicated.
8. ☐ Allowable subject matter having been indicated, formal drawings are required in response to this Office action.
9. ☐ The corrected or substitute drawings have been received on _____. These drawings are ☐ acceptable; ☐ not acceptable (see explanation).
10. ☐ The ☐ proposed drawing correction and/or the ☐ proposed additional or substitute sheet(s) of drawings, filed on _____ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).
11. ☐ The proposed drawing correction, filed _____, has been ☐ approved. ☐ disapproved (see explanation). However, the Patent and Trademark Office no longer makes drawing changes. It is now applicant's responsibility to ensure that the drawings are corrected. Corrections MUST be effected in accordance with the instructions set forth on the attached letter "INFORMATION N HOW TO EFFECT DRAWING CHANGES", PTO-1474.
12. ☐ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. _____; filed on _____.
13. ☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
14. ☐ Other

PTOL-326 (Rev. 7-82) EXAMINER'S ACTION

EXHIBIT R

Claims 40-52 stand withdrawn from further consideration by the examiner, 37 CFR 1.142(b), as being drawn to a non-elected invention, the requirement having been traversed in Paper No. 8.

This application has been filed with informal drawings which are acceptable for examination purposes only. Formal drawings will be required at the time the application is allowed.

The specification is objected to under 35 U.S.C. 112, first paragraph, as failing to provide an enabling disclosure. This paragraph of the statute requires that the specification shall contain a written description of the invention and of the manner and process of making and using it, in such full, clear, concise and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention. This objection and the following rejection are repeated essentially for reasons given in the last Office action paragraph bridging pages 2-3 and last paragraph on page 3. Applicant's arguments (paper no. 14, pages 2-6) are not persuasive because each of the references relied upon by applicant deals only with polynucleotides and the issue here has to do with oligo-

nucleotides. Furthermore, the passage in the Stebbing (page 303) article referred to by applicant (paper no. 14, page 4) says nothing about uptake of polynucleotides by cells. Also, the discussion in Stebbing (Cell Biol. Int. Repts. vol. 3) on pages 493-496 is deemed to support this objection and the following rejection. Befort et al shows (page 184) that antiviral activity is "limited only to fragments longer than 40 to 50 nucleotides"; again supporting this objection and the following rejection.

Claims 1-4, 6-15, 18-25, 27-37, 39 and 53 are rejected under 35 U.S.C. 112, first paragraph, for the reasons set forth in the objection to the specification. This rejection is repeated essentially for reasons given in the last Office action (last paragraph on page 3).

Claims 1-4, 6-15, 18-25, 27-37, 39 and 53 are rejected under 35 U.S.C. 112, first paragraph, as the disclosure is enabling only for claims limited in accordance with the disclosure at pages 1-20 of the specification. See MPEP 706.03(n) and 706.03(z). This rejection is repeated essentially for reasons given in the last office action (first full paragraph on page 4). The discussion concerning RNAs in the above rejection is incorporated here. It is noted that applicant has not argued the point regarding the synthesis of RNAs.

Claims 14, 16, 17, and 27 are rejected under 35

U.S.C. 112, first and second paragraphs, as the claimed invention is not described in such full, clear, concise and exact terms as to enable any person skilled in the art to make and use the same, and/or for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Claims 16 and 17 are incomplete in that there is no antecedent basis for "said linker sequence" (claim 16) or "the linker" (claim 17). This part of this rejection can be overcome by amending claims 16 and 17 to depend from claim 15 as it is believed was originally intended. Claim 27 is incomplete in that there is no antecedent basis for the phrase "said hybridization".

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1 and 3-19 are rejected under 35 U.S.C. 103 as being unpatentable over Itakura et al in view of either one of Paterson et al or Hastie et al. This rejection is repeated essentially for reasons given in the last Office action (paragraph bridging pages 6-7). Applicant's arguments (paper no. 14, pages 7-10) are not persuasive. Applicant should note that not all of the claims require the inhibition of protein synthesis, are not limited to the use of oligoribonucleotides, and do not require that any oligonucleotide be introduced into cells.

Claims 1, 3-28, 30-39, and 53 are rejected under 35 U.S.C. 103 as being unpatentable over Itakura et al in view of either one of Paterson et al or Hastie et al as applied to claims 1 and 3-19 above, and further in view of any one of Pluskal et al, Pitha (CRC Press), Befort et al, Arya et al (Molec. Pharamacol. or BBRC), Summerton, Tennant et al, Miller et al (Biochem. 16: 1988), Stephenson et al, Zamecnik et al, or Stebbing et al. This rejection is repeated essentially for reasons given in the last Office action (paragraph bridging pages 8-9). Applicant's arguments (paper no. 14, pages 12-15) are not persuasive. It is noted that applicant acknowledges Zamecnik et al and Stephenson et al to have achieved inhibition of protein synthesis using a tridecamer. Applicant argues that it would not be obvious to use an oligonucleotide complementary to the coding

region of an mRNA to achieve hybrid arrested translation and cites work (e.g. Holder and Lingrel) showing the translation of mRNA with a high degree of secondary structure. However, the claims are not limited to the use of RNA oligomers. In fact, there is no showing in the instant application that oligoribonucleotides work and it appears that applicant has argued effectively that they do not.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a). The practice of automatically extending the shortened statutory period an additional month upon the filing of a timely response to a final rejection has been discontinued by the Office. See 1021 TMOG 35.

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION IS NOT MAILED UNTIL AFTER THE END OF THE THREE-MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED, AND ANY EXTENSION FEE PURSUANT TO 37 CFR 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

Any inquiry concerning this communication should be directed to J. Martinell at telephone number 703-557-3920.

Martinell:wcg

4/25/85
retyped 5/1/85

THOMAS G. WISEMAN
SUPERVISORY PATENT EXAMINER
ART UNIT 127

APPENDIX J

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET N
06/314,124	10/23/81	TULLIS	22178

FULWIDER, PATTON, RIEBER, LEE & UTECHT
3435 WILSHIRE BLVD., STE. 2400
LOS ANGELES, CA 90010

Kindly Acknowledge Receipt
GGK

RECEIVED
DEC 02 1985

EXAMINER	
MARTINELL, J	
ART UNIT	PAPER NUMBER
127	18

DATE MAILED: 11/27/85

This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

FULWIDER, PATTON, RIEBER, LEE & UTECHT

Due: February 27, 1986

☒ This application has been examined ☐ Responsive to communication filed on _____ ☐ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), _____ days from the date of this letter. Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☒ Notice of References Cited by Examiner, PTO-892.
2. ☐ Notice re Patent Drawing, PTO-948.
3. ☐ Notice of Art Cited by Applicant, PTO-1449
4. ☐ Notice of informal Patent Application, Form PTO-152
5. ☐ Information on How to Effect Drawing Changes, PTO-1474
6. ☐ _____

Part II SUMMARY OF ACTION

1. ☒ Claims 1, 3-28, and 31-53 are pending in the application.
   Of the above, claims 40-52 are withdrawn from consideration.
2. ☒ Claims 2 and 29 have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☒ Claims 1, 3-28, 31-39, and 53 are rejected.
5. ☐ Claims _____ are objected to.
6. ☐ Claims _____ are subject to restriction or election requirement.
7. ☒ This application has been filed with informal drawings which are acceptable for examination purposes until such time as allowable subject matter is indicated.
8. ☐ Allowable subject matter having been indicated, formal drawings are required in response to this Office action.
9. ☐ The corrected or substitute drawings have been received on _____. These drawings are ☐ acceptable; ☐ not acceptable (see explanation).
10. ☐ The ☐ proposed drawing correction and/or the ☐ proposed additional or substitute sheet(s) of drawings, filed on _____ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).
11. ☐ The proposed drawing correction, filed _____, has been ☐ approved. ☐ disapproved (see explanation). However, the Patent and Trademark Office no longer makes drawing changes. It is now applicant's responsibility to ensure that the drawings are corrected. Corrections MUST be effected in accordance with the instructions set forth on the attached letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474.
12. ☐ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. _____; filed on _____.
13. ☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
14. ☐ Other

EXHIBIT J

The finality of the Office action mailed May 8, 1985 is withdrawn.

Claims 40-52 are withdrawn from further consideration by the examiner, 37 CFR 1.142(b), as being drawn to a nonelected invention, the requirement having been traversed in Paper No. 8.

This application has been filed with informal drawings which are acceptable for examination purposes only. Formal drawings will be required when the application is allowed.

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The specification is objected to under 35 U.S.C. 112, first paragraph, as failing to provide an enabling disclosure.

Save

This objection and the following rejection are repeated essentially for reasons already of record (e.g. Office action mailed May 8, 1985 paragraph bridging pages 2-3 and first full paragraph on page 3. Applicant's arguments (paper No. 14, pages 2-6) are not per-

suasive because each of the references relied upon by applicant deals only with polynucleotides and the issue here has to do with oligonucleotides. Furthermore, the passage in the Stebbing (page 303) article referred to by applicant (paper No. 14, page 4) says nothing about uptake of polynucleotides by cells. Also, the discussion in Stebbing (Cell Biol. Int. Repts. vol. 3) on pages 493-496 is deemed to support this objection and the following rejection. Befort et al shows (page 184) that antiviral activity is "limited only to fragments longer than 40 to 50 nucleotides"; again supporting this objection and the following rejection. Limiting the claims to the use of oligodeoxyribonucleotides as proposed by applicants (see attachment to Interview Summary Record of October 18, 1985) would overcome this objection and the following rejection.

Claims 1-4, 6-15, 18-25, 27-37, 39, and 53 are rejected under 35 U.S.C. 112, first paragraph, for the reasons set forth in the above objection to the specification.

Claims 1-4, 6-15, 18-25, 27-37, 39, and 53 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. This rejection is repeated

APPENDIX K

I hereb tify that this correspondence is being deposited with the ..ited States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231, on 4/1/86
Date

By Cathryn Campbell
Cathryn Campbell, Reg. No. 31,815

4/1/86
Date of Signature

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Richard H. Tullis

Serial No. 06/314,124

Filed: October 23, 1981

For: OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHOD OF MAKING SAME

Group Art Unit: 127

Examiner: J. Martinell

Los Angeles, CA 90010
March 31, 1986

AMENDMENT

Hon. Commissioner of Patents
and Trademarks
Washington, D. C. 20231

Sir:

Responsive to the Office Action mailed November 27, 1985, please amend the above-identified application as follows:

IN THE CLAIMS

Cancel claims 1, 3-28, and 30-39 without prejudice. Substitute therefor the following claims:

--54. A method of developing oligodeoxyribonucleutide therapeutic agents for use in in vivo inhibition of the synthesis of one or more targeted proteins in a cell without substantially inhibiting the synthesis of non-targeted proteins, comprising the steps of:

EXHIBIT K

determining the base sequence of an organism's messenger ribonucleic acid, said base sequence coding for at least a portion of said protein targeted for inhibition;

synthesizing an oligodeoxyribonucleotide, the nucleotide sequence of which is substantially complementary to at least a portion of said base sequence, and

at least a portion of said oligodeoxyribonucleotide being a more stable form in order to limit degradation in vivo, whereby said oligodeoxyribonucleotide may be introduced into the cells of said organism for hybridization with said messenger ribonucleic acid base sequence coding for at least a portion of a protein targeted for inhibition so as to substantially block translation of said base sequence and inhibit synthesis of said targeted protein.--

--55. The method of claim 54, wherein said more stable form is a phosphotriester form.--

--56. The method of claim 54, wherein said oligodeoxyribonucleotide comprises at least 14 nucleotides.--

--57. The method of claim 54, wherein said oligodeoxyribonucleotide comprises about 23 nucleotides.--

--58. The method of claim 54, wherein the order of said base sequence is determined from ribonucleic acid or

deoxyribonucleic acid coding for said targeted protein prior to synthesizing the oligodeoxyribonucleotide.--

--59. The method of claim 54, wherein the order of said base sequence is determined from messenger ribonucleic acid coding for said targeted protein prior to synthesizing said oligodeoxyribonucleotide.--

--60. The method of claim 54, wherein the order of said base sequence is determined from said targeted protein prior to synthesizing said oligodeoxyribonucleotide.--

--61. The method of claim 54 further comprising the step of inserting said oligodeoxyribonucleotide into a plasmid for cloning.--

--62. The method of claim 61, wherein said plasmid is pBR322.--

--63. The method of claim 62, wherein said oligodeoxyribonucleotide is inserted into said plasmid with a linker base sequence.--

--64. The method of claim 63, wherein said linker base sequence is GATTCGAATC or CTAAGCTTAG.--

--65. The method of claim 63, wherein said linker base sequence is susceptible to partial degradation by Hind III or Alu I restriction nucleases.--

--66. The method of claim 54, wherein said oligodeoxyribonucleotide is synthesized chemically.--

--67. The method of claim 54 further comprising the step of:

cross-hybridizing the oligodeoxyribonucleotide against messenger ribonucleic acid from at least one species different from said organism, and selecting that fraction of the oligodeoxyribonucleotide which does not so hybridize so as to increase the specificity of the selected oligodeoxyribonucleotide to messenger ribonucleic acid unique to said organism.--

--68. A method of selectively inhibiting in vivo synthesis of one or more specific targeted proteins without substantially inhibiting the synthesis of non-targeted proteins, comprising the steps of:

synthesizing an oligodeoxyribonucleotide having a nucleotide sequence substantially complementary to at least a portion of the base sequence of messenger ribonucleic acid coding for said targeted protein,

at least a portion of said oligodeoxyribonucleotide being a more stable form to limit degradation in

vivo;

introducing said stable oligodeoxyribonucleotide into a cell; and

hybridizing said stable oligodeoxyribonucleotide with said base sequence of said messenger ribonnucleic acid coding for said targeted protein, whereby translation of said base sequence is substantially blocked and synthesis of said targeted protein is inhibited.--

--69. The method of claim 68, wherein said oligodeoxyribonucleotide comprises at least 14 nucleotides.--

--70. The method of claim 68, wherein said oligodeoxyribonucleotide comprises about 23 nucleotides.--

--71. The method of claim 68, wherein said targeted protein is follicle stimulating hormone, which has an alpha chain and a beta chain.--

--72. The method of claim 71, wherein the oligodeoxyribonucleotide comprises the nucleotide sequence $GTGTAGCAGTAR_1CCR_2GCGCACCA$, and wherein R_1 is G or T and R_2 is G or T.--

--73. The method of claim 68, wherein said

hybridization occurs at about 37°C.--

--74. The method of claim 68, wherein said oligodeoxyribonucleotide is formed through diester bonding.--

--75. The method of claim 68, wherein said more stable form is a phosphiotriester form.--

--76. A method of controlling the infection of a host organism by a foreign organism through the selective inhibition of the synthesis of a protein vital to the foreign organism's viability, comprising the steps of:

determining the base sequence of the foreign organism's nucleic acid, said base sequence coding for at least a portion of said protein vital to the foreign organism's viability;

synthesizing an oligodeoxyribonucleotide the order of nucleotides being substantially complementary to a portion of the foreign organism's messenger ribonucleic acid coding for said protein vital to said foreign organism's viability,

at least a portion of said oligodeoxyribonucleotide being a more stable form to inhibit degradation in vivo;

introducing said oligodeoxyribonucleotide into the cells of said host organism; and

hybridizing said oligodeoxyribonucleotide with said portion of the foreign organism's messenger ribonucleic acid

so as to substantially block translation of said foreign organism's messenger ribonucleic acid coding for said protein, thereby inhibiting synthesis of said protein vital to the viability of the foreign organism.--

--77. The method of claim 76, wherein said more stable form is a phosphotriester form.--

--78. The method of claim 76 further comprising the step of:

determining the order of the base sequence of said organism's nucleic acid prior to synthesizing the oligodeoxyribonucleotide.--

--79. The method of claim 76 further comprising the step of:

cross-hybridizing the oligodeoxyribonucleotide against messenger ribonucleic acid from at least one species different from said foreign organism and selecting that fraction of the oligodeoxyribonucleotide which does not so hybridize so as to increase the specificity of the selected oligodeoxyribonucleotide against said foreign organism.--

--80. The method of claim 79, wherein said cross-hybridization is performed against messenger ribonucleic acid from said host rganism.--

--81. The method of claim 79, wherein the selected oligodeoxyribonucleotide substantially hybridizes only with a messenger ribonucleic acid unique to said foreign organism.--

--82. A genetically engineered therapeutic process which inhibits synthesis of one or more targeted proteins within the cells of an organism without substantially inhibiting synthesis of non-targeted proteins, comprising the steps of:

determining the base sequence of the messenger ribonucleic acid coding for the targeted protein;

synthesizing an oligodeoxyribonucleotide having a nucleotide sequence substantially complementary to the region of the messenger ribonucleic acid coding for said targeted protein,

at least a portion of said oligodeoxyribonucleotide being a more stable form to inhibit degradation in vivo;

introducing said oligodeoxyribonucleotide into the cells of said organism; and

hybridizing said oligodeoxyribonucleotide with said base sequence of said messenger ribonucleic acid coding for said targeted protein, whereby translation of said base sequence is substantially blocked and synthesis of said targeted protein is inhibited.--

REMARKS

This application has been carefully reviewed in light of the Office Action dated November 27, 1985. In that Office Action, the Examiner withdrew the finality of the Office Action mailed May 8, 1985, in light of newly cited art. The Specification was objected to and claims were rejected under 35 USC 112 and/or 35 USC 103 or 35 USC 102(e). In response, claims 1-39 have been cancelled and claims 54-82 substituted therefor. In this connection, revisions to the claims conform to those submitted to the Examiner in an interview on October 23, 1985, and reflect a number of helpful suggestions made by the Examiner at that time.

The Examiner's time and attention with respect to this application is greatly appreciated. Applicant wishes to particulary thank the Examiner for his courtesies during the recent interview, at which time various aspects of the previous Office Action were discussed. Those present at the interview of October 23, 1985, were Examiner Martinell, the applicant, Mr. Richard Tullis; his representatives, Mr. Gilbert Kovelman and Ms. Cathryn Campbell; and Mr. Vincent Frank, President of Molecular Biosystems, Inc., the assignee of the present application. At the interview, the invention was discussed in detail, and a proposed set of new claims, conforming to those hereinafter introduced by amendment, were provided to the Examiner.

As the new claims were intended to avoid points raised by the Examiner in the previous Office Action, they were discussed in some detail. In particular, the claims

were limited to deoxyribonucleotides, which limitation the Examiner stated to have obviated previous rejections under 35 USC 112 which should be withdrawn. Moreover, the claims have been limited to oligodeoxyribonucleotides binding to mRNA sequences in the coding region so as to premit inhibition of synthesis of specific targeted proteins. The Examiner indicated that he was favorably impressed with this argument as distinguishing over the prior art. In light of the fact that the Examiner introduced new art, viz Miller et al. U. S. Patent No. 1,151,713, the finality of the previous rejection was withdrawn.

The latest Office Action again includes rejections duplicating those of the previous Office Action, which rejections were discussed at the Interview of October 23, 1985. In a recent telephone interview, the Examiner indicated to Ms. Campbell that the rejections were merely repeated for formal reasons.

In the latest Office Action, claims 1-4, 6-15, 18-25, 27-37, 39, and 53 were rejected, and the Specification objected to under 35 USC 112, first paragraph, as failing to provide an enabling disclosure. Specifically, it was asserted that the application does not provide evidence of the introduction of oligoribonucleotides into the cytoplasm of the cells with which it is contacted, as it must in order to form the RNA-RNA hybrids as allegedly specified and claimed.

As applicant has stated in previous papers, it is his belief that such uptake of oligoribonucleotides is clearly confirmed by various references, most notably Befort. In any event, however, the rejected claims have been

cancelled and the new claims have been limited to oligodeoxyribonucleotides in accordance with the suggestion of the Examiner in the Office Action of May 8, 1985. This ground of rejection is thus believed to have been obviated. Moreover, the Examiner indicated at the Interview of October 23, 1985, that the newly cited Miller reference, U. S. Patent No. 1,151,713, corroborates uptake of oligonucleotides by cells.

Claims 1-4, 6-15, 18-25, 27-37, 39, and 53 were rejected under 35 USC 112 as the specification is asserted not to be enabling for RNA-RNA hybridization. As noted above, the rejected claims have been cancelled. The substituted claims are limited to deooxyribonucleotides and, thus, it is again believed that this ground of rejection is obviated.

Claims 14, 16, 17, and 27 were rejected under 35 USC 112, second paragraph, for lacking antecedent basis for certain claims. Again, in light of the amendment herein, these informalities have been overcome.

Claims 1 and 3-19 were rejected under 35 USC 103 as being unpatentable over Itakura et al. in view of either Paterson et al. or Hastie et al. All claims were also rejected under 35 USC 103 in view of the above references and also in view of any one of Pluskal et al, Pitha (CRC Press) , Befort et al., Arya et al. (Molec. Pharmacol. or BBRC), Summerton, Tennant et al., Miller et al. (Biochem. 16:1988), Stephenson et al., Zamecnik et al., or Stebbing et al. The Examiner has previously alleged that Itakura discloses the synthesis of oligonucleotides and the secondary and tertiary references disclose inhibition of protein synthesis and the

introduction of oligonucleotides into cells. In his Amendment dated December 4, 1984, applicant has previously presented references and arguments indicating that the present invention is not rendered obvious by the cited references. Applicant will not repeat this material here in light of the present amendment which restricts all claims to oligodeoxyribonucleotides which are introduced into cells so as to inhibit protein synthesis. As has been previously stated, neither alone nor in combination do the cited references disclose or suggest the introduction of oligonucleotides complementary to specific portions of the coding region of an organism's mRNA so as to inhibit synthesis of the particular targeted proteins. Moreover, in the claims, as now amended, these restrictions are explicit, an argument with which the Examiner indicated his favorable impression at the Interview of October 23, 1985.

All claims have been rejected under either 35 USC 102 or 35 USC 103 on the basis of newly cited art, namely U. S. Patent No. 4,469,863 to Ts'o et al. (It is assumed that the Office Action's listing of claim 43 was a typographical error, and should have read claim 53). The Examiner alleges that Ts'o teaches specific inhibition of protein synthesis by using oligonucleotides complementary to mRNA. Applicant respectfully traverses this ground of rejection.

The Ts'o reference involves synthetic oligonucleotide aryl and alkyl phosphonates for use primarily in intracellular binding to transfer RNA anticodons or to transfer RNA amino acyl acceptor stems. Transfer RNA's so bound at the anticodon site are either unable to reach amino

acids or are unable to recognize and bind to the complementary codon of the messenger RNA and thus protein synthesis is allegedly inhibited.

While the reference purports to teach that such tRNA binding can be used to accomplish specific inhibition of a selected nucleotide sequence so as, for example, to inhibit the growth of tumor cells of viruses, the examples specifically teach away from the use of these oligonucleotides to inhibit in vivo protein synthesis, as taught specifically in the present application.

Ts'o tested the effect of short homooligo-A deoxyadenosine (d-A_{2-4}) methyl phosphonate in bacterial and mammalian cells and cell-free systems. In the cell free system, while some effect was noted on synthetic poly-A and poly-U messages, significantly no effect was observed on the translation of globin mRNA. The rabbit beta-globin polypeptide has some 3 phenylalanine residues coded for by the triplet U-U-U. In addition, there are seven other positions in beta-globin mRNA to which the tetrameric methyl phosphonate should bind. Therefore, were Ts'o's oligonucleotides truly capable of combining with the message so as to inhibit translation, an effect on globin synthesis would be observed. The fact that the inventors detected no such effect suggests that, in fact, these oligonucleotides are incapable of blocking translation of the message. Moreover, the fact that significant effects were observed on synthetic poly-A as well as poly-U mRNA suggests some sort f competitive interaction with tRNA rather than specific hybridization with the homopolymer template.

In testing their methyl phosphonate oligonucleotides

on mammalian cells, Ts'o was able to establish uptake, as taught in the present application. Further, inhibitory effects on colony formation were observed in both bacterial and mammalian cells in culture. However, significantly, the inventors note at Column 26, lines 5-7 that "no inhibitory effects in cellular protein or DNA synthesis could be detected in the presence of [the stabilized oligonucleotides] by the present assay procedures."

Several explanations for the effect of the methyl phosphonate oligonucleotides are proposed. As Ts'o states at Column 27, lines 17-19, their observations "suggest that inhibition [of cell-free aminoacylation] occurs as a result of oligomer binding to the -- -U-U-U -- anticodon loop of the tRNA." It is noted that the results presented are kinetically consistent with interaction with the anticodon loop sequence. Moreover, because of evidence that the anticodon loop of the lysine tRNA in E. coli is related to the synthetic recognition site, the effect may derive from interference with the coupling of the amino acid to the corresponding transfer RNA.

Thus, it is apparent that in contrast to the Examiner's suggestion that methyl phosphonate oligonucleotides in Ts'o could be utilized to specifically control and inhibit protein synthesis in vivo, the experimental evidence presented in the Ts'o specification actually teaches away from such a use. While the data is consistent with methyl phosphonate oligonucleotides binding anticodons, nowhere do the results indicate that the oligonucleotides are selectively binding with the portion of the mRNA coding for specific proteins. In fact, the data in

Ts'o evidencing lack of inhibition of globin synthesis directly indicate that such messenger inhibition is not occurring.

Parenthetically, in Column 28, lines 24-28, the patent refers to "forming complexes with the poly (U) message." Because, as indicated above, there is no suggestion that any inhibition observed was due to binding of the methyl phosphonate oligonucleotides to the poly-U template, the word "message" in this context must refer only to the homopolymer template, which is in no way equivalent to naturally occurring messenger RNA. This distinction is forcefully shown by the clear cut lack of inhibition of globin synthesis, the only actual "messenger" against which he tested the methyl phosphonates.

In contrast to the teachings of Ts'o, the present application involves oligodeoxyribonucleotides, the nucleotide sequence of which is chosen so as to bind specifically with the nucleotide sequence of mRNA in order to effect inhibition of the translation of specific targeted proteins. Because of the short length of the methyl phosphonates disclosed in Ts'o and their evident lack of effect on natural mRNA, these compounds are not capable of providing a mechanism of selective inhibition of protein synthesis. In fact, the Ts'o reference clearly states at Column 25, lines 8-9, that there was no indication whatsoever of translation inhibition in vivo. Therefore, the Ts'o reference, far from anticipating the present application or rendering it obvious, teaches away from the invention.

In summary, applicant points out that the cited art does not provide a basis for concluding that the present

invention is anticipated or obvious. Further, applicant has amended the claims in accordance with the discussion at the recent interview with the Examiner so as to overcome the Examiner's previous grounds of rejection. In light of the foregoing, it is respectfully believed that reconsideration and allowance of all the applicant's claims is in order.

Respectfully submitted,

FULWIDER, PATTON, RIEBER, LEE & UTECHT

Cathryn Campbell
Registration No. 31,815
Attorney for Applicant

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APPENDIX L

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
07/140,718	12/29/87	TULLIS R	100321/0054

PRETTY, SCHROEDER, BRUEGGEMANN AND CLARK
444 SOUTH FLOWER STREET, SUITE 2000
LOS ANGELES, CA 90071

RECEIVED
MAR 27 1989
P.S.B.C.-S.D.

EXAMINER	
MARTINELL, J	
ART UNIT	PAPER NUMBER
185	42

DATE MAILED: 03/22/89

RECEIVED
MAR 27 1989
Pretty, Schroeder, Brueggemann & Clark

Below is a communication from the EXAMINER in charge of this application
COMMISSIONER OF PATENTS AND TRADEMARKS

ADVISORY ACTION

Resp. May 14 '89

☒ THE PERIOD FOR RESPONSE:

☒ is extended to run 4 months from the date of the Final Rejection

☐ continues to run ________ from the date of the Final Rejection

☐ expires three months from the date of the final rejection or as of the mailing date of this Advisory Action, whichever is later. In no event however, will the statutory period for response expire later than six months from the date of the final rejection.

Any extension of time must be obtained by filing a petition under 37 CFR 1.136(a), the proposed response and the appropriate fee. The date on which the response, the petition, and the fee have been filed is the date of the response and also the date for the purposes of determining the period of extension and the corresponding amount of the fee. Any extension fee pursuant to 37 CFR 1.17 will be calculated from the date that the shortened statutory period for response expires as set forth above.

☐ Appellant's Brief is due in accordance with 37 CFR 1.192(a).

☒ Applicant's response to the final rejection, filed 03/07/89 has been considered with the following affect, but it is not deemed to place the application in condition for allowance:

1. ☐ The proposed amendments to the claim and/or specification will not be entered and the final rejection stands because:

a. ☐ There is no convincing showing under 37 CFR 1.116(b) why the proposed amendment is necessary and was not earlier presented.

b. ☐ They raise new issues that would require further consideration and/or search. (See Note).

c. ☐ They raise the issue of new matter. (See Note).

d. ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal.

e. ☐ They present additional claims without cancelling a corresponding number of finally rejected claims.

NOTE: ____

2. ☐ Newly proposed or amended claims ________ would be allowed if submitted in a separately filed amendment cancelling the non-allowable claims.

3. ☒ Upon the filing of an appeal, ~~xxxxxxxxxxxxxxxxxxxxxxxxxxxx~~ the status of the claims in this application would be as follows:

Allowed claims: None

Claims objected to: None

Claims rejected: 54, 56-60, 66-74, 76, and 78-82

However;

a. ☐ The rejection of claims ________ on references is deemed to be overcome by applicant's response.

b. ☐ The rejection of claims ________ on non-reference grounds only is deemed to be overcome by applicant's response.

4. ☐ The affidavit, exhibit or request for reconsideration has been considered but does not overcome the rejection.

5. ☐ The affidavit or exhibit will not be considered because applicant has not shown good and sufficient reasons why it was not earlier presented.

☐ The proposed drawing correction ☐ has ☐ has not been approved by the examiner.

☒ Other

See page 2.

EXHIBIT L

The objection and rejection under 35 USC 112 (Office action mailed November 14, 1988, last two paragraphs on page 2) stand for reasons already of record. Applicant has not responded to this objection and rejection.

The rejection under 35 USC 102(e) or in the alternative under 35 USC 103 over Ts'o et al ('863) (Office action mailed November 14, 1988, paragraph running from pages 3 through 5) is withdrawn in view of the Declaration Under 37 CFR 1.131 filed March 7, 1989. It has been presumed that the exhibits referred to in the declaration by Roman numerals are actually the exhibits accompanying the declaration that are labeled with Arabic numerals.

The rejection under 35 USC 103 (Office action mailed November 14, 1988, first full paragraph on page 3 stands essentially for reasons already of record. Applicant's arguments (paper no. 40) are not convincing because Miller et al discloses oligonucleotide ethyl phosphotriesters to be resistant to degradation by nucleases (e.g., see Miller et al at page 1988, first paragraph of the text of the article). Applicant's argument in connection with Miyoshi et al (the "Miyake" referred to by applicant?) is unconvincing because Miyoshi et al is cited only to show synthesis of longer oligonucleotides to be routine. The argument that Miller et al does not teach the invention is unconvincing because the rejection is under 35 USC 103, not 35 USC 102.

Any inquiry concerning this communication should be directed to J. Martinell at telephone number (703) 557-0664.

Martinell
03/21/89

JAMES MARTINELL, Ph.D.
Examiner
Art Unit 127 185

APPENDIX M

PATENT
Our Docket: P31 8026

RESPONSE UNDER 37 CFR 1.116
EXPEDITED PROCEDURE
EXAMINING GROUP 185

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:
Richard H. Tullis

Serial No.: 140,916

Filed: December 29, 1987

For: OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHOD OF MAKING SAME

Group Art Unit: 185

Examiner: J. Martinell

Los Angeles, CA 90071
March 3, 1989

RESPONSE

Hon. Commissioner of Patents
and Trademarks
Box AF
Washington, D.C. 20231

Sir:

This amendment is submitted in response to the November 14, 1988 Office Action and the December 9, 1988 Communication from the Examiner. Applicants have requested a one month extension of time. Therefore, a response is due March 14, 1989.

REMARKS

Applicant respectfully requests the Examiner reconsider and withdraw the various grounds of rejection set forth in the November 14, 1988 Office Action and D cember 9, 1988 Communication.

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231, on 3-3-89
Date
By Cathy Campbell, Reg. #31,015
3-3-89
Date of Signature

EXHIBIT M

In the December 9, 1988 communication from the Examiner, applicant's Declaration Under 37 C.F.R. 1.31 submitted with a November 21, 1988 Preliminary Amendment was alleged improper. Applicants submit herewith a Supplemental Declaration Under 37 C.F.R. 1.31 by Richard H. Tullis, the inventor of the subject application.

The enclosed Supplemental Declaration and the attached Exhibits establish that the present invention was conceived prior to November 12, 1980, the effective date of the T'so Patent as a reference under 35 U.S.C. 102(e) or 103. Moreover, the attached Exhibits demonstrate the diligence of Applicant and his attorney in pursuing filing the application. As Exhibits IV to VII indicate, the application went through a number of revisions including extensive modifications. As will be appreciated, the present invention was developed during a period when the technology relating to the use of oligonucleotides was in its infancy.

The subject matter of the above-identified application and its parent application is quite technologically complex. At the time that the original application was filed, October 21, 1981, biotechnology patent protection was in its infancy. The decision in Diamond vs Chakrabarty, which set the stage for claiming biotechnological innovations, came down on June 16, 1980. Patent attorneys were scrambling to understand the full impact of this decision. Moreover, the above-identified application, being a pioneering effort in the field, required considerably more attention to such issues as providing an enabling disclosure and accurately claiming the invention than would an application in a more mature technology.

Thus, Applicant submits that pursuant to 35 C.F.R. 1.31 the date of Applicant's conception of the invention prec ded

that of the filing of the T'so reference, and that the inventor and his attorneys diligently pursued filing of the application. The reference should therefore be removed.

In the November 14, 1988 Office Action, the Examiner continued the rejection of all claims as obvious under 35 U.S.C. 103 over the combinations of references to Itakura, Paterson, Hastie, Summerton and Miller. Applicant vigorously traverses these grounds of rejection. As will be noted from the prosecution history, Applicant has repeatedly distinguished over the teachings for which the Examiner has cited these combined references. For example, in the Amendment filed April 4, 1986, all claims were limited to deoxyribonucleic acids.

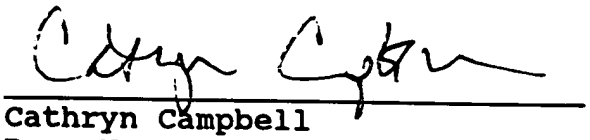
The Examiner has once again cited these references, emphasizing now the teaching of Miller which allegedly teaches inhibition by oligonucleotides longer than the trimers actually utilized by Miller. As Applicant has previously pointed out, any such "teaching" is merely speculation, particularly in light of Miller's concurrent teaching that making longer oligonucleotides is not feasible. The Examiner has asserted that the production of such longer oligonucleotides "has long been routine in this art." (Office Action mailed June 11, 1987.) Were such oligonucleotides within the purview of those skilled in the art at the time in question, Applicant would pose the question: Why did Miller not utilize such longer oligonucleotides?

The reference to Miyake cited merely as interest, is alleged to teach the synthesis of oligonucleotides. In fact, Miyake, although utilizing the phosphotriester <u>method</u> of oligonucleotide synthesis, does not teach the synthesis of phosphotriesters. On page 3637, line two, he refers to the

removal of p-chlorophenyl. Moreover, he indicates that the product of the synthesis was partially digested with phosphodiesterase, unlike the product of the present invention which is resistant thereto. Applicant maintains his position that, if the synthesis of stabilized oligonucleotidase such as phosphotriesters was known to those skilled in the art, Miller, who was undoubtedly skilled in the art, would have employed such oligonucleotides, rather than merely speculating as to their effects.

For the foregoing reasons, Applicant believes that the claims in the above-identified test are in condition for allowance and urges that a notice to this effect be forthcoming.

Respectfully submitted,



Cathryn Campbell
Reg. No. 31,815

PRETTY, SCHROEDER,
BRUEGGEMANN & CLARK
444 South Flower Street
Suite 2000
Los Angeles, California 90071

APPENDIX N

PATENT
Our Docket: P31 8026

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Richard H. Tullis Parent Ser. No.: 140,916 Parent Filed: December 29, 1987 For: OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME	Group Art Unit: Examiner: J.Martinell Los Angeles, CA 90071 February 8, 1989

DECLARATION UNDER 37 C.F.R. 1.131

Hon. Commissioner of
Patent & Trademarks
Washington, D.C. 20231

Sir:

I, Richard H. Tullis, residing at 1320 Saxony Road, Leucadia, California 92024, declare as follows:

1. I am the inventor who, on October 23, 1981, filed an application for United States Letters Patent entitled, Oligonucleotide Therapeutic Agent and Method of Making Same, which application was given Serial No. 314,124. On information and belief, based on statements made to me by Cathryn Campbell, attorney of record of the above-identified application, Serial No. 140,916 is a Continuation of Serial No. 002,014, which was, in turn a Continuation of Serial NO. 314,124.

2. The invention disclosed and claimed in Serial No. 314,124, and now claimed in Serial No. 140,916, was conceived

by me in this country prior to November 12, 1980. Attached in support of conception prior to this date are Exhibits I through III, attached hereto. Although the dates on the Exhibits have been obliterated, I have reviewed them and all predate November 12, 1980.

3. Exhibit I is a copy of a letter from me to William M. Smith, Esq., then associated with the firm of Fulwider, Patton, Rieber, Lee & Utecht, and one of the attorneys with whom I interfaced in the preparation and filing of the application. It was dated by me prior to November 12, 1980. The letter sets forth the essential elements of the invention in sufficient detail to permit one skilled in the art to practice the invention without undue experimentation. On the last page, it states that "this idea first occurred to me.....in the afternoon of [a date preceding November 12, 1980]."

4. Exhibit II is a copy of a letter from me to William Smith, Esq. which was dated by me and stamped as received by the Fulwider firm. Both dates predate November 12, 1980. The letter discloses the invention. On information and belief, based on statements made to me by Cathryn Campbell, attorney of record in the pending patent application, it was the policy of the Fulwider firm that correspondence received by the firm was stamped with the date received. Correspondence was then attached to the appropriate file. The letter expands at length on the specific details of th invention.

5. With diligence, I proceeded to file an application on the invention. Because of the complexity of the subject matter of the inventor~~or~~ion, RCH I had extensive and continuing interaction with the attorneys preparing the application, including personal meetings, telephone calls and meetings. On information and belief, based on statements made to me by

Cathryn Campbell, attorney of record in the pending patent application, the files in her possession contain evidence of the diligence of the attorneys and the inventor in filing the original application. Such evidence includes documents indicating extensive interaction between the attorneys and the inventor including memos of telephone conversations, indications of personal meetings, and drafts of applications. Various of these documents are identified and submitted herewith as Exhibits IV through VII.

6. Exhibit IV is a copy of a letter dated prior to November 12, 1980, from me to William Smith, Esq., which accompanied disclosure materials and instructed Mr. Smith to "fire away." Certain material unrelated to the date of the letter has been obliterated.

7. Exhibit V is a copy of a letter from me to William Smith, Esq. dated February 4, 1981, accompanying a first draft of the patent. I indicated therein that much work on the patent "remains to be done." Certain material unrelated to the date has been obliterated.

8. Exhibit VI is a copy of a letter from me to William Smith, Esq., dated February 23, 1981, accompanying literature related to the invention. Certain material unrelated to the date has been obliterated.

9. Exhibit VII is a copy of a letter from me to William Smith, Esq., dated June 1, 1981, accompanying a revised draft of the application and indicating that it is still incomplete.

10. On information and belief, based on statements made to me by Cathryn Campbell, attorney of record in the pending patent application, the file contains other letters, documents and telephone memos indicating a continuing

interaction between me and Mr. Smith relating to the preparation and filing of the application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

Dated: FEBRUARY 9, 1989

Richard H. Tullis

RICHARD H. TULLIS

Enc:

Ex I - Letter
Ex II - Letter
Ex III - Information Sheet
Ex IV - Letter
Ex V - Letter
Ex VI - Letter
Ex VII - Letter